STUDY OF RECAPTURED PRIMARY DATA IN JOURNAL ARTICLES CONCERNING THE SOD1 G93A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

A Thesis
Presented to
The Academic Faculty

by

Renaid B. Kim

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STUDY OF RECAPTURED PRIMARY DATA IN JOURNAL ARTICLES CONCERNING THE SOD1 G93A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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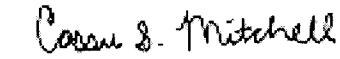

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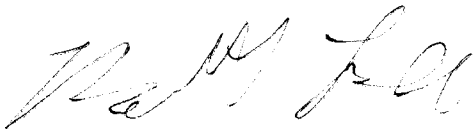

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CHAPTER 1

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a debilitating disease that is characterized by late-onset, progressive neuronal degeneration of the motor neurons, which results in dysphagia, dyspnea, dysarthria and eventually death. There is currently no cure for ALS, with riluzole being the only FDA-approved drug that only extends survival by 3 to 6 months without repairing the damaged motor neurons. Transgenic mice, especially the one with superoxide dismutase 1 glycine 93 to alanine mutation (SOD1-G93A), have been a mainstay in the study of ALS and its underlying pathophysiology, with more than 3,000 articles upon a Pubmed search of (Amyotrophic Lateral Sclerosis OR ALS) AND (G93A OR Mouse).

Such a wealth of data allows for informatics approaches to understanding the pathophysiology of ALS. To form a framework for conducting an informatics study with primary journal articles about ALS, a nine-category ontology was created via a comprehensive, keyword-based agnostic survey of more than 1,300 journal articles, which is presented in Chapter 2. A meta-analysis of 45 journal articles was conducted to examine temporal relationships between calcium homeostasis, mitochondrial dysfunction and oxidative stress and to elucidate the timing of the disturbances, which is presented in Chapter 3. Chapter 4 thoroughly describes the biocuration process used for the projects in the laboratory and presents interesting findings about the associates in the laboratory.

CHAPTER 2

STATE OF THE FIELD: AN INFORMATICS-BASED SYSTEMATIC REVIEW OF THE SOD1-G93A AMYOTROPHIC LATERAL SCLEROSIS TRANSGENIC MOUSE MODEL

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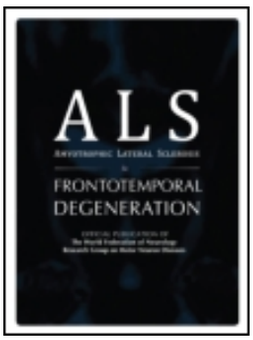

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ORIGINAL ARTICLE

State of the field: An informatics-based systematic review of the SOD1-G93A amyotrophic lateral sclerosis transgenic mouse model

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Abstract

Numerous sub-cellular through system-level disturbances have been identified in over 1300 articles examining the superoxide dismutase-1 guanine 93 to alanine (SOD1-G93A) transgenic mouse amyotrophic lateral sclerosis (ALS) pathophysiology. Manual assessment of such a broad literature base is daunting. We performed a comprehensive informatics-based systematic review or 'field analysis' to agnostically compute and map the current state of the field. Text mining of recaptured articles was used to quantify published data topic breadth and frequency. We constructed a nine-category pathophysiological function-based ontology to systematically organize and quantify the field's primary data. Results demonstrated that the distribution of primary research belonging to each category is: systemic measures an motor function, 59%; inflammation, 46%; cellular energetics, 37%; proteomics, 31%; neural excitability, 22%; apoptosis, 20%; oxidative stress, 18%; aberrant cellular chemistry, 14%; axonal transport, 10%. We constructed a SOD1-G93A field map that visually illustrates and categorizes the 85% most frequently assessed sub-topics. Finally, we present the literature-cited significance of frequently published terms and uncover thinly investigated areas. In conclusion, most articles individually examine at least two categories, which is indicative of the numerous underlying pathophysiological interrelationships. An essential future path is examination of cross-category pathophysiological interrelationships and their co-correspondence to homeostatic regulation and disease progression.

Key words: *Mitochondria, excitotoxicity, gliosis, protein aggregation, reactive oxygen species, rotarod, calcium, neuropathology*

Introduction

Amyotrophic lateral sclerosis (ALS) is characterized by progressive neurodegeneration of the motor neurons, which leads to muscle paralysis, respiratory deficiency, and eventually death. Mutations of the superoxide (copper-zinc) dismutase-1 (SOD1) gene have been identified as contributors to familial ALS, which accounts for approximately 5–10% of all ALS cases (1). The SOD1-G93A (glycine 93 to alanine) mutation is a comparatively rare ALS mutation in humans, but it is the most studied and published mutation within experimental transgenic ALS mouse models (2,3). The SOD1-G93A transgenic ALS model's popularity is largely due to its ALS symptom reproducibility and its widespread availability for purchase from The Jackson Laboratory (jaxmice.jax.org). At the end of the 2014 year, searching for 'Amyotrophic Lateral Sclerosis' AND 'G93A' in PubMed returned approximately 1300 articles, and the tally was actually greater since not every article using the SOD1-G93A model specifically cites 'G93A' in the PubMed-searchable locations (i.e. title, abstract, etc.).

The SOD1-G93A mouse model has been utilized to identify numerous deficits and impairments contributing to or the direct result of the mutation's associated ALS pathophysiology. Briefly, such deficits comprise the following: apoptosis, including changes in pro- and anti-apoptotic signals (4); axonal transport of mitochondria and other key cargoes (5); aberrant cellular chemistry such as reduced enzyme activity and metal mishandling (6); energetics, including disturbances of the physical and functional properties of mitochondria, ATP production and calcium homeostasis (7); genetic damage, including changes in mRNA or DNA; inflammation, including the migration of reactive astrocytes and microglia (8); oxidative stress, resulting from the build-up of free radicals (9); proteomics, characterized by the accumulation of misfolded SOD1 aggregates (10);

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systemic impairments, including overall system-level neuromuscular function and non-neuromuscular contributors (3). The phenotype severity and disease progression of the SOD1-G93A transgenic mouse is largely dependent upon the transgene copy number (typically denoted as 'high' versus 'low'). The overwhelming majority of SOD1-G93A transgenic mouse studies have used a high copy model, which has an average onset range of 85–100 days and endpoint of 120–160 days (3).

Knowing the distribution and categorization of primary data is a key step towards both consolidating current knowledge and planning new research (11). However, with so many articles covering such an expansive and complex pathophysiology, it is difficult to manually determine what has and has not been examined in the SOD1-G93A transgenic ALS mouse field. Moreover, while traditional literature reviews help in digesting the details of published data, ideas, or mechanisms, their content does not necessarily quantitatively align with what actual primary data exist for a given topic or theorem. Authors of traditional literature reviews must subjectively determine what topics are reported based on the author's exposure to the field. Automated informatics-based systematic reviews or 'field analyses' overcome the traditional limitations of manual literature reviews by comprehensively and agnostically searching the primary data of every available article to quantify the breadth and depth of researched topics. The result is an objective map of the overall literature that structurally organizes and numerically identifies topical areas of prevalent data as well as disparate or thinly investigated areas where primary data are sparse.

Consequently, the goals of this informatics-based systematic review of the SOD1-G93A mouse model were to: 1) determine the published breadth and frequency of research topics; 2) systematically organize and categorize primary data articles using a pathophysiological function ontology; and 3) consolidate current knowledge and highlight corresponding future research paths.

Materials and methods

The general method included finding SOD1-G93A articles; recapturing data from the article entities; devising and testing a term-category dictionary for identifying research terms/topics and for ontological categorization; searching the article entities to determine the frequency of primary data terms/research topics; assessment of the publication frequency and distribution within the pathophysiological ontological categories.

Inclusion and exclusion criteria

To obtain the initial primary article selection pool, PubMed searches were conducted in October 2014 to find all published articles with ('Amyotrophic Lateral Sclerosis' or 'ALS') in the title or abstract and ('transgenic mouse' or 'G93A') in the title or abstract. Initial primary article selection pool exclusion criteria consisted of: non-English language articles; articles for which full-text pdf downloads were unavailable; and articles labeled as literature reviews. Articles were either downloaded using PubMed Central or from e-journal subscriptions available from the libraries of Georgia Institute of Technology and Emory University. Using these methods, less than 3% of the eligible initial article pool was unavailable for download. Based on these initial criteria, 1997 articles were eligible for inclusion in the initial primary article selection pool.

To obtain the final article pool utilized to conduct this study, within-article keyword searches were performed to find articles that contained 'G93A' in at least one of the following locations: article title, abstract, figure caption, or within the figure text (see Data recapture for details). The final article pool consisted of 1339 articles, all of which were included in the field analysis.

It should be noted that the overwhelming majority of studies do not distinguish between strain and transgene copy number in the searchable article entities (and many articles do not mention them at all, even in the full-text methods). Thus, articles were not included or excluded based on transgene copy number (e.g. high, low) or strain (e.g. B6SJL, C57BL/6), i.e. our article pool represents a combined assessment of research topics in the overall SOD1-G93A transgenic mouse field.

Data recapture

Data were recaptured from the following article locations, referred to as entities: article title, abstract, figure captions, and within figure text. 'Within figure' text included any text labeled on or within a figure or table, e.g. the x-y axis labels, bar graph categorical labels, legends, etc. Recaptured data were obtained from downloaded full-text pdf files. Abstract, title, and reference information was exported directly from PubMed. Figure captions and within figure text was manually scraped from the full-text pdf articles using a standard keyboard copy and paste command (12). Any special characters that did copy correctly were manually revised. A quality control team independently assessed all data recapture to ensure complete accuracy. Recaptured data were transcribed into a custom project-specific searchable relational database (www.pathology-dynamics.org). The database is implemented in Filemaker 13 Pro Advanced (Filemaker, Inc.).

Term-category dictionary

A dictionary of corresponding terms and categories (referred to as a term-category dictionary) was

constructed that assigned frequent SOD1-G93A pathophysiology article terms and phrases to their most probable ontological category. The term-category dictionary allowed for automated searching of recaptured text and labeling of the articles entity's and the overall article's most probable ontological categories.

The chosen ontological categories were based on a previously published scheme (2) developed from a meta-analysis of SOD1-G93A traditional literature review articles. The ontology was used to categorize primary research data based on underlying pathophysiological function. The ontological categories consisted of: Apoptosis, Axonal Transport, Chemistry, Energetics, Excitability, Genetic Damage, Inflammation, Oxidative Stress, Proteomics and Systemic. The ontological categories are defined in detail in the Results and Discussion section.

To determine the most frequent keywords and phrases in the SOD1-G93A articles, word and phrase frequency analysis was performed using freely available software from WriteWords. Approximately 6500 different terms and phrases were identified and sorted by their number of appearances in each recaptured entity and by their total number of appearances in the articles. Non-scientific words insignificant to the analysis (e.g. of, in, and, etc.) were immediately excluded. Subsequently, a group of trained researchers in SOD1-G93A pathophysiology preliminarily labeled the most frequent 2000 terms by their most likely ontological category.

After performing the first ontological test set search, some individual terms were combined to provide for better specificity (see details in Ontological Test sets). Ultimately, 670 terms and phrases were selected for inclusion of the term-category dictionary (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1047455).

Field searches

Each keyword or phrase in the term-category dictionary was searched in the recaptured data article entities (article title, abstract, figure caption, and within figure text). If the search keyword was a single word, a whole word search was performed. For a phrase, a whole word search was performed for each word but not necessarily in the order of the words, e.g. 'copper concentration' and 'concentration of copper' were detected upon searching for 'copper concentration'. If the search was positive, the figure and article was labeled by the corresponding ontological category of that term. The categories identified in the figure caption and within figure text were combined to represent each individual figure's ontological categorization. The categories identified in the article title, figure caption, and within figure text were combined to represent the overall categorization of each article. It should be noted that the abstract was ultimately excluded from the overall article categorization due to the number of false-positive hits it produced (see Test sets). Similarly, the ontological category, Genetic Damage, was individually excluded from the final field analysis results to decrease false-positives; corresponding articles were re-categorized according to the cited location of genetic damage (see Test sets).

Test sets

Test sets of SOD1-G93A figures and corresponding articles were constructed to determine which recaptured article entities should be searched and to evaluate the term-category dictionary. Each test set minimally consisted of 500–600 figures from 100 different SOD1-G93A articles, which included primary data representing each ontological category. For the purpose of evaluating the term-category dictionary, the test set's ontological categorization was separately and manually determined by independent visual inspection of the article's primary data by five trained SOD1-G93A pathophysiology researchers.

Evaluation included measures of sensitivity and specificity. Sensitivity (the ability of a test to identify a condition correctly) and specificity (the ability of a test to exclude a condition correctly) are often used to assess the capability of a search to produce accurate results. Sensitivity is defined as: number of true-positives (TP) divided by the sum of the number of true-positives and false-negatives (FN): TP/ [TP + FN]. Specificity is defined as the number of true-negatives divided by the sum of true-negatives and false-positives: TN/[TN + FP]. While having both a high sensitivity and specificity is ideal, realistically optimization is typically favored towards one or the other depending on the search/test outcome goal, i.e. whether it is more important that the search/test includes or excludes a condition. Given that our protocol searches multiple terms per entity and thus allows multiple categories to be assigned, specificity (the ability to exclude) was given greater priority in the test set design and assessment.

We assessed the article entities' ability to correctly represent the primary data contained within the article. Searching the abstract text resulted in $>50\%$ false-positives, i.e. over 50% of the abstracts contained key terms or phrases that were either not represented/relevant to the article's primary data or were not present in the article's figure caption or within figure text. If the abstracts were to be used as part of the determination of the articles' overall categorization, their false-positive terms would result in the addition of non-relevant categories. In contrast, searching the figure captions and within figure text resulted in $<2\%$ false-positives and article titles $<4\%$. Therefore, as noted in field searches, the abstract search was excluded from the final ontological categorization of an article.

Subsequently, we assessed the ability of each ontological category to represent the corresponding article's primary data. All categories had greater than 95% specificity with the exception of Genetic Damage. The category Genetic Damage consisted of many general terms (see Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1047455), which resulted in low specificity (<70%). Fortunately, genetic damage is typically measured in a specific location, organelle or pathway. Thus, articles containing primary data that examined genetic damage were labeled with the category(ies) that corresponded to the location or physiology affected by the genetic damage (e.g. mitochondrial mRNA damage → Energetics).

Finally, an ontological test set was also utilized to assess the term-category dictionary itself. An initial test to identify false-positive and negatives resulted in 86.1% sensitivity and 79.0% specificity for articles and 81.5% sensitivity and 87.6% specificity for figures. Care was taken to increase specificity by combining terms that created numerous false-positives into more specific phrases (e.g. aggregation → protein aggregation). After correction, another test set was utilized to evaluate the final dictionary's accuracy: 87.9% sensitivity and 99.9% specificity for papers and 77.9% sensitivity and 97.5% specificity for figures. Given that specificity was the priority, an overall specificity >98% was considered acceptable for the study goals.

Results and discussion

We first present the field analysis results, including the overall distribution of research articles with primary data belonging to each of the nine ontological categories: Apoptosis, Axonal Transport, Chemistry, Energetics, Excitability, Inflammation, Oxidative Stress, Proteomics and Systemic. Subsequently, the quantitative topical distribution of research articles in each of the individual categories is presented and explained. Finally, we conclude with a discussion on categorical relationships and future directions.

Overall SOD1-G93A Field Analysis

We performed a field analysis based on key word searches of article titles, figure captions, and within figure text to examine the prevalence of the different types of pathophysiological research in the SOD1-G93A ALS transgenic mouse model. The order of overall prevalence of primary data corresponding to the defined ontological categories of the SOD1-G93A ALS published literature is as follows: 1) systemic and functional measures, 59%; 2) inflammation, 46%; 3) cellular energetics, 37%; 4) proteomics, 31%; 5) excitability, 22%; 6) apoptosis, 20%; 7) oxidative stress,18%; 8) aberrant cellular chemistry, 14%; and 9) axonal transport, 10%. Figure 1 shows the distribution of articles across the nine categories as a percentage of the total SOD1-G93A articles along with their absolute article count.

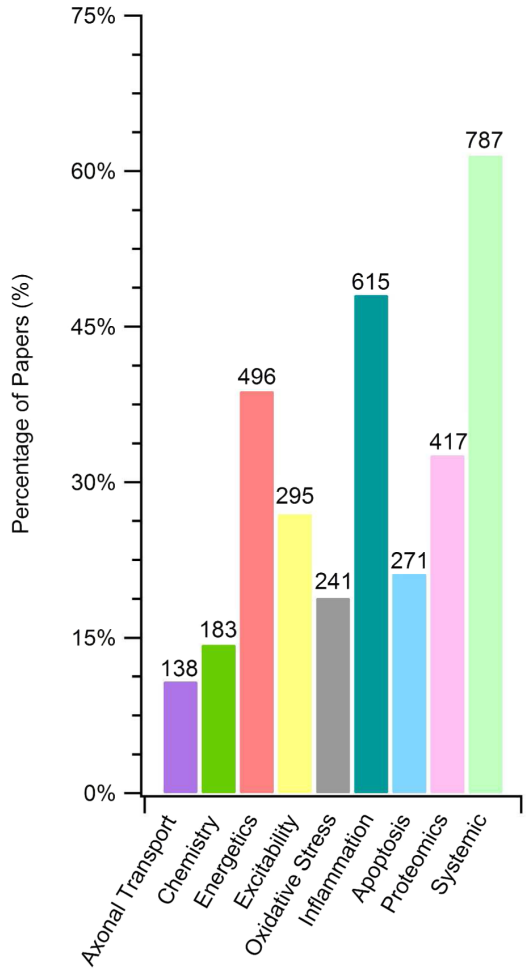

Figure 1. SOD1-G93A transgenic mouse model research categorized by article topic frequency based on a nine-category pathophysiological function ontology. For each category, the graph illustrates the absolute article count and percentage of the total SOD1-G93A articles with primary data based on word frequency searches of the title, figure captions, and within figure text. Articles are typically classified under two or three categories due to the many inherent biological and pathological inter-category relationships.

All included primary articles and their figures were labeled with at least one category using the term-category dictionary. However, given the complexity of the SOD1-G93A pathology, most articles actually belonged to more than one category. For example, a primarily proteomic study examining protein aggregation in conjunction with iron sulfur protein (ISP) belongs to both chemistry (due to 'iron') and proteomics (due to 'protein aggregation'). The average number of categories labeled for each article using the term-category dictionary search was 2.6 with a standard deviation of ± 1.6. Multi-category assignment to SOD1-G93A articles is, in large part, due to the numerous cross-category pathophysiological relationships (see Categorical relationships and future directions).

The most common 12 terms of each category (with the variations 'or'-merged into one search) accounted for the categorization of greater than 85% of the articles in the category. Thus, these most prevalent 12 terms per category were used to develop the field analysis map shown in Figure 2. The field analysis map visually illustrates the proportion of the SOD1-G93A primary data encompassed by each overall category and also the most prevalent within category terms. The size of the boxes corresponds to the approximate relative size of the categories or the categorical terms. In Table I we reveal the number of articles and figures/tables with primary data corresponding to each of the 12 most prevalent terms per category. The full term-category dictionary and field analysis assessment is shown in Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1047455.

Topical Analysis within Ontological Categories

We present the informatics results of the topical analysis performed within each of the ontological categories and provide brief explanations of the topics' significance to the SOD1-G93A transgenic mouse pathophysiology. Given the goals of this informatics-based systematic review, the primary purpose of the following text is to quantify and expound upon the preponderance of articles and sub-topics included in each ontological category. For further in-depth discussion of mechanisms and published experimental study results, we refer the reader to the cited references or topic-specific traditional literature reviews.

Apoptosis. Apoptosis, representing 21% of the SOD1-G93A transgenic ALS mouse literature, encompasses all programmed cell death signaling pathways. Apoptosis has multiple relationships with other ontological categories given that the ultimate endpoint of the ALS pathology is cell death.

Caspases, and in particular caspase 1, 3, and 9, are responsible for many of the signaling cascades (13) that initiate apoptosis and, as such, are the most represented term under this category (122 articles, 45% of Apoptosis). Intracerebroventricular administration of zVAD-fmk, a broad caspase inhibitor, has been shown to delay disease onset and mortality in SOD1 ALS mice (13). Other key signals include Bcl-2 (31 articles, 11% of Apoptosis), which has both pro- and anti-apoptotic mechanisms, and Bax (25 articles, 9% of Apoptosis), which is pro-apoptotic (4,14). Many studies have examined the neuroprotective effects of Bcl-2 and how, in abundance, it

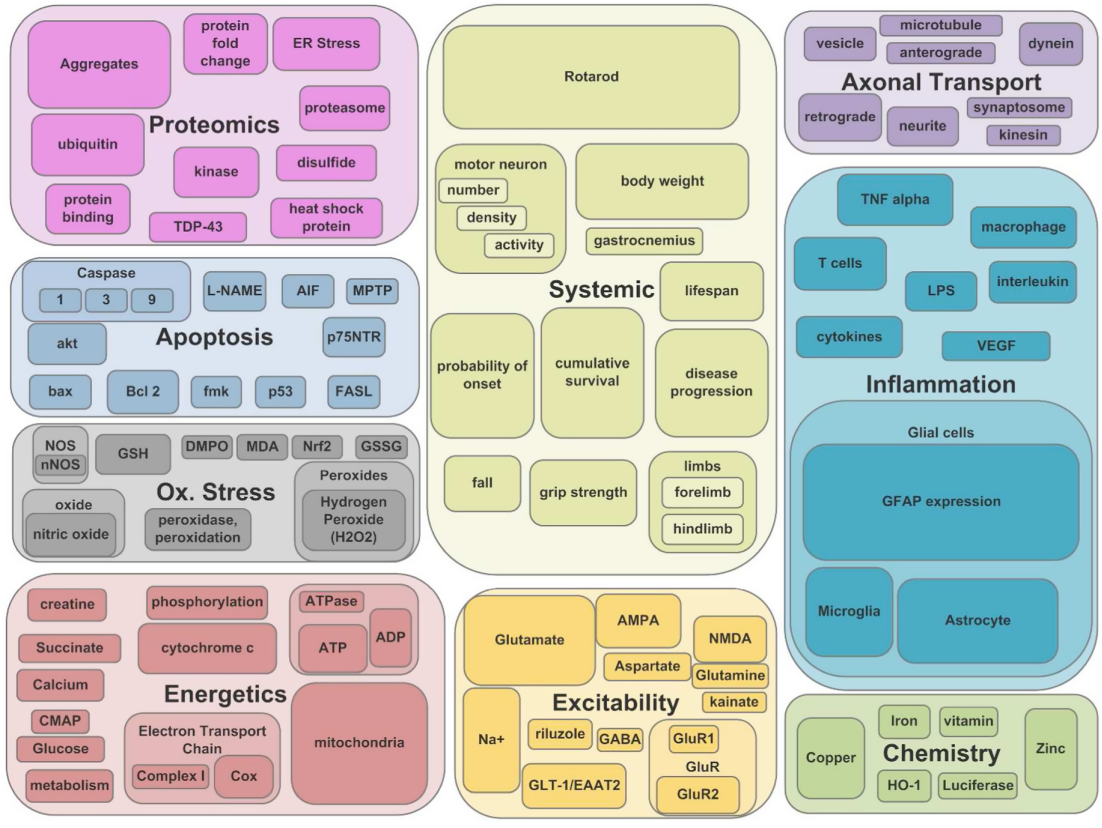

Figure 2. Field map of the most prevalent SOD1-G93A transgenic mouse research topics. The sizes of the boxes represent the relative term frequency. The map illustrates categorical terms required to encompass at least 85% of the articles classified to each of the nine categories: Apoptosis, Axonal transport, Chemistry, Energetics, Excitability, Inflammation, Oxidative stress, Proteomics, and Systemic.

Table I. The 12 most common terms or phrases per category are presented with a description and the resultant number of articles (A) and figures (F). Note that the 12 terms provided more than 85% coverage in each category (see Test sets).

Axonal Transport	A	F	Description/Significance
retrograde, retrogradely	44	96	The movement of dynein cargoes towards the cell body
neurite, neurites	38	53	A projection from the cell body, so an axon or dendrite.
dynein	20	84	The motor protein that is responsible for carrying cargoes retrogradely
microtubule, microtubules	17	38	Neural structure or "tracks" on which dynein and kinesin travel.
vesicle	17	38	Transport unit often carried by dynein and kinesin.
anterograde, anterogradely	16	40	The movement of kinesin cargoes from soma towards neuromuscular junction
Synaptosome(s)	11	52	A type of vesicle transported in the axon
axon terminal, projection	10	13	Catch-all terms for detecting axonal transport papers.
kinesin	7	15	The motor protein that is responsible for carrying cargoes anterogradely
loa	7	28	"Legs at Odd Angles" - mutation that affects dynein "legs" (25).
neurofilament* transport	5	16	Neural structure element carried via "slow" axonal transport.
wallerian	4	9	Wallerian Degeneration is the degeneration of an axon.
Chemistry	**A**	**F**	**Description/Significance**
copper, Cu2+	58	236	Used by SOD1
zinc, Zn2+	53	216	Used by SOD1
metal, metals	35	92	Catch-all terms for detecting data discussing metals
luciferase	31	41	An oxidative enzyme used in assays
iron, Fe, Fe2+	17	44	Involved in Fenton reaction, which produces hydroxyl radical
HO-1, Heme oxygenase	17	40	Enzyme that catalyzes the degradation of heme, producing iron
vitamin, B12	16	48	Tried as a treatment (32).
lithium	8	46	Tried as a treatment (33).
ferritin	8	14	Controls iron in a cell
NaHCO3	5	10	Used as a buffer in various experiments, particularly those assessing metalation
Salubrinal	4	11	Used to suppress SOD1 activity (89).
VPA	4	9	Valproic acid. Tried as a treatment (34).
Energetics	**A**	**F**	**Description/Significance**
mitochondrial, mitochondria, mito, mitochondrion,	194	773	Produce ATP, involved in cellular respiration & calcium homeostasis.
calcium, Ca2, Ca2+, ca	100	238	Required for respiration, excitability, and muscle contraction.
Cox, complex IV	80	135	Last enzyme in the respiratory electron transport chain
ATP, ADP, ATPase	74	129	Energy units made or used in cellular respiration
cytochrome, cytochrome c	69	141	Transfers electrons from complex III – IV
phosphorylation	47	89	Process of adding a phosphate group to a protein, turning it "on" or "off"
metabolism, metabolic	43	80	General term encompassing energy harvesting
complex I	28	51	Element of the electron transport chain of mitochondrial respiration
glucose	25	45	Main "fuel" for cellular respiration
creatine	23	75	Increases the formation of ATP
succinate	23	35	Encompasses Succinate Dehydrogenase, aka complex II, role in respiration
depolarization	19	37	Measure of mitochondrial respiration
Excitability	**A**	**F**	**Description/Significance**
Glutamate	108	401	Excess extracellular glutamate causes neuronal degeneration
GLT1, GLT-1, EAAT2, EAAT	56	188	Glutamate transporters; decreases observed in ALS patients and G93A mice
Na, Na+	52	112	Required for action potential; ions enter cell upon activation of glutamate receptors
Excitotoxicity, excitatory,	48	137	Terms describing toxic over-excitation
AMPA	35	107	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid- glutamate receptor mediating fast excitatory transmission
cmap, cmaps	35	53	Compound muscle action potential-summation of several action potentials over several muscle fibers in one area
riluzole	33	85	Sodium channel blocker; the only FDA-approved drug for ALS.
NMDA	27	43	N-methyl-D-aspartate- glutamate receptor mediating slow excitatory transmission
GABA	22	50	ϒ-Aminobutyric acid- chief inhibitory neurotransmitter in mammals
GluR*, GluR1, GluR2, GluR	20	60	Glutamate receptors: Down-regulation of GluR2 leads to excess Ca2 + influx
glutamine	16	34	Neurologically inactive form - precursor - of glutamate
aspartate	16	32	Stimulates NMDA receptors, but not as strongly as glutamate does
Oxidative stress	**A**	**F**	**Description/Significance**
H2O2, Hydrogen peroxide	69	129	Free radical. Relatively weak but can produce stronger oxidants.
oxide	47	154	Catch-all term for elements causing oxidation
ROS, reactive oxygen species	47	90	Reactive molecules containing oxygen. Source of free radicals.
GSH, glutathione	45	101	Antioxidant that prevents damage from free radicals.
nitric, nitric oxide	43	145	Also known as NO. Common type of free radical.
Peroxidase, Peroxidation	36	84	Assist in oxidative degradation; activity is enhanced in ALS mice

(*Continued*)

Table I. (Continued)

nNOS, NOS	34	68	Produces NO upon stimulation by inflammatory cytokines
Peroxide, peroxides	26	44	Catch-all term for any peroxide. See hydrogen peroxide.
MDA	15	20	Marker for oxidative stress
Nrf2	15	73	Involved in antioxidant and anti-inflammatory defense (6).
GSSG	14	19	Oxidized form of glutathione
DMPO	11	27	Used in spin trapping to measure the levels of free radicals.
Inflammation	**A**	**F**	**Description/Significance**
GFAP	245	399	Glial fibrillary acidic protein-an indicator of the progression of gliosis
astrocyte	233	579	Astroglia that support neural function; assist in scarring following neural damage
T-cells, T-cell, CD11b, CD4	181	331	Provides neuroprotection
microglia, microglial	172	406	Excess activation leads to neurodegeneration
glial cell, glia	106	247	Catch-all terms for various glial cells.
inflam*, neuroinflamm*, immune	79	285	Catch-all terms for neuroinflammatory processes.
TNF, TNF alpha, TNFalpha, TNFa, TNF a, tumor necrosis factor	66	129	Tumor necrosis factor α. Stimulates immune activation, leading to gliosis
IL, IL 6, IL 4, interleukin	56	78	Interleukins stimulate immune cell activation, leading to gliosis
macrophage, M2, M1	53	88	Regulate immune activity via production of cytokines
VEGF	44	124	Vascular endothelial growth factor. Tried as a treatment (69)
cytokine, cytokines	38	82	Catch-all terms microglial priming and immune cell activation elements
LPS	33	56	Lipopolysaccharide- activates glial cells, inducing gliosis
Apoptosis	**A**	**F**	**Description/Significance**
caspase	122	225	Key apoptotic signal
apoptosis, apoptotic	114	294	Programmed cell death
akt	43	81	When activated by VGEF possibly acts as an anti-apoptotic factor
Bcl 2	31	85	A protein involved in both pro and anti-apoptotic mechanisms
bax	25	43	A pro-apoptotic protein
fmk	19	27	Anti-apoptotic signaling pathway
p75NTR	17	44	p75 neurotrophin receptor promotes caspase-dependent axon degeneration
p53	17	38	Tumor-suppressing protein in apoptosis.
L-NAME	15	28	Reduces NO and reverts the pro-apoptotic factors of it
FASL	11	26	Fas ligand; induces apoptosis when bound to its receptor
MPTP	10	27	Neurotoxin that induces apoptosis
XIAP	9	15	X-linked inhibitor of apoptosis protein
Proteomics	**A**	**F**	**Description/Significance**
Aggregate(s), aggregation,	168	575	Aggregates of mutant, misfolded proteins are a hallmark of ALS
ubiquitin	89	165	Affects protein degradation, trans-location, and interaction
Kinase(s)	65	193	Affects protein activity, signaling; implicated in protein aggregation in ALS
protein binding	58	109	Catch-all term for binding processes of mutant SOD1 leading to aggregation.
proteasome, proteasomal	54	143	Involved in protein degradation.
disulfide	35	131	Disulfide bonds mediate the aggregation process in SOD1
oligomer*	30	40	Catch-all term for proteins
heat shock protein, hsp	30	74	Response to proteomic stress. Up-regulation extends survival.
ER Stress	27	75	Endoplasmic reticulum stress from unfolded proteins
misfolded protein, protein fold change	27	43	The fold change (misfolded proteins) leads to protein aggregates.
protein degradation	24	36	Dysfunctional degradation of misfolded proteins causes aggregates
TDP-43, TDP 43	23	59	TAR-DNA binding protein 43. Inclusions commonly found in ALS patients.
Systemic	**A**	**F**	**Description/Significance**
Density, count or activity of motor neurons (all spellings)	323	483	Includes functional measures of locomotor activity and assessment of motor neuron degeneration
rotarod, rotorod, rot*rod	217	267	Experimental device and test used to assess mouse motor function
disease progression	205	553	Catch-all term for in vivo observation of disease progression.
hindlimb, forelimb, limb,	173	318	Hindlimb tremors are commonly used as a marker of disease onset
body weight	171	233	Indicator of disease progression; decreases in later stages of the disease.
lifespan, life span	121	240	The total time spent alive for the subject.
cumulative survival	103	119	The endpoint of the disease. Total lifespan of subject.
probability of onset	102	109	The time when symptoms of ALS typically begin to appear.
grip, grip strength	94	134	Test assessing mouse's ability to grip; indicator of motor function/progression
in vivo, invivo	88	209	"Within the living" - encompasses all experiments performed on live test subjects.
gastrocnemius	75	151	Large muscle found in the hindlimb, easy to access and evaluate.
fall	59	73	The action of falling down, usually due to inability to stand.

could be used to abolish the proapoptotic component of Bax in SOD1-G93A mice (4,15).

P53 and p75NTR have been examined equally with 17 articles each, collectively representing 13% of the Apoptosis literature. An increased level of p53 tumor protein is observed in ALS patients (16), but the absence of p53 does not affect the SOD1-G93A mice (17,18). p75NTR is a neurotrophin receptor that regulates signal cascades and functions of cells, and has been implicated in motor neuron degeneration in ALS (19). Reduction of Fas ligands (FASL) was examined in 11 articles as a way to increase survival in ALS mice (20). MPTP, examined in 10 articles, is a neurotoxin known to induce apoptosis that has been shown to increase SOD1 activity when administered to SOD1-G93A mice (21).

Axonal Transport. Comprising just 10% of the SOD1-G93A literature, axonal transport has been the least studied pathophysiological category. Molecular motors carry necessary constituents in the axon from the soma to the neuromuscular junction (i.e. anterograde transport via kinesin) and from the neuromuscular junction to the soma (i.e. retrograde transport via dynein) (22,23). Mutations to the machinery and cargoes can impair their attachment to the motor proteins and their mobility (5). Notably, in SOD1-G93A transgenic ALS mice, axonal transport deficits appear well before cell degeneration occurs (24). In addition to possible transport-specific defects, axonal transport is thought to be further hindered due to inadequate mitochondrial ATP (see Energetics), an over-abundance of misfolded SOD protein aggregates (see Proteomics), and possible excitotoxic burdens (see Excitability).

Retrograde movement was the term that appeared most often in the axonal transport literature (44 articles, 23% of Axonal Transport) with dynein ranking third in frequency (20 articles, 14% of Axonal Transport). A mutation in dynein has been shown to rescue axonal transport defects and overall extend the lifespan of ALS SOD1-G93A mice (25). Comparatively, anterograde transport (16 articles, 12% of Axonal Transport) by kinesin (seven articles, 5% of Axonal Transport) does not appear as frequently in the SOD1-G93A literature despite both anterograde and retrograde transport deficits having been documented in SOD1-G93A mice (26).

Chemistry. The ontological category of chemistry, accounting for 14% of the SOD1-G93A literature, includes measures of aberrant cellular chemistry, enzymatics, catalytics and metal mishandling present in the SOD1-G93A ALS pathophysiology (2). Copper and zinc collectively represent 39% of the chemistry literature, although their frequency is slightly over-represented due to their appearance in the name of 'copper zinc superoxide dismutase-1'. Beyond their involvement in SOD1, copper and zinc concentrations have been measured in different locations, with decreases shown in the liver and spinal cord (27). The effect of zinc supplementation in SOD1-G93A mice has been examined, including its increased affect on NMDA-mediated excitotoxicity in SOD1, as well as its negligible impact on survival (28).

Another frequent cellular chemistry assessment is iron homeostasis, which represents 9% of the chemistry literature. Iron homeostasis has been shown to be impaired in both SOD1-G93A transgenic mice and in human ALS patients (29). An increase in iron content and iron genes expression has been observed in G93A-SOD1-transfected neuroblastoma cells compared to wild-type counterparts (6). Iron could also be contributing to the disease via the Fenton reaction, which accelerates hydroxyl radical production that damages cellular DNA (30).

Heme oxygenase-1 (HO-1), representing 9% of the chemistry literature, is an enzyme that assists in degrading heme, and has been observed to increase as ALS progresses (31). Vitamins (32), lithium (33) and valproic acid (VPA) have been explored (34) as treatment options but have shown negligible success.

Energetics. Energetics, which encompasses mitochondrial production of ATP via cellular respiration, is the third most represented ontological category, encompassing 39% of the SOD1-G93A transgenic mouse literature. Understandably, mitochondria and variations of this word are the most represented terms, encompassing 39% of the Energetics category itself. As ALS progresses, mitochondrial ability to produce ATP decreases (35), which leads to axonal transport deficiencies, axonal retraction, denervation, and death of cells via apoptosis (36). SOD1-G93A mice mitochondria change in both physical appearance and chemical functionality as the disease progresses (37).

Calcium, the second most prevalent Energetics term, represents 20% of the Energetics ontological category. Calcium overload, a known issue in SOD1-G93A mice, leads to cell death via increased membrane permeability and loss of ATP production. Overexpression of Ca^{2+} binding proteins such as parvalbumin (38) and calbindin D28K (38,39) have been shown to improve disease parameters. Interestingly, most articles examining calcium homeostasis utilize in vitro assessments (40), although in vivo examination is increasing (41). Finally, it should be noted that while calcium is listed in Energetics because the majority of articles examining it investigate calcium handling by mitochondria (42), there are also other aspects of calcium that are clearly related to excitability due to its role in neural transmission and axonal transport (41).

Glucose and related cellular pathway machinery encompasses the remainder of the most prevalent

keywords in the Energetics category, as shown in Table I. Specifically, glucose utilization rates are impaired in SOD1-G93A mice in as early as 60 days (43). GAPDH, an enzyme important for the breakdown of glucose for energy, as well as creatine, have also been found to decrease by approximately 40% in SOD1-G93A mouse models (44). Creatine treatment has been shown to protect against excitotoxic lesions created by NMDA (45) and MPTP, which interferes with complex I, slowing mitochondrial metabolism. Finally, complex I, pyruvate, and cytochrome C have all have shown some forms of deficiency in SOD1-G93A mice (35).

Excitability. Excitability is ontologically defined as the physiological pathways involved in producing action potentials. Excitability is an integral aspect of the ALS pathophysiology, representing 23% of the SOD1-G93A transgenic mouse literature. More specifically, excitability encompasses excitotoxicity, the pathological toxic over-excitation of neurons that is thought to contribute to the neuronal degeneration seen in SOD1-G93A ALS mice (46). However, there has been recent debate as to whether SOD1-G93A mice experience hyperexcitability (47), hypoexcitability (48) or a combination of both that changes with temporal disease progression (2).

Over-excitation due to glutamate homeostasis is the most frequently cited excitotoxic contributor (160 + articles, 54% of Excitability). Most of the SOD1-G93A specific research has focused on glutamate uptake, concentrations of glutamate in various locations and their effects on the systemic progression of the disease. Since overstimulation is caused by the influx of sodium (56 articles, 18% of Excitability) and calcium ions, various treatments have been tried to inhibit the voltage-gated sodium and calcium channels. Riluzole (12% of Excitability literature), one of the most common treatments for clinical ALS, is thought to work by inactivating the voltage dependent sodium receptors (49,50) on the glutamatergic nerve terminals (46,51).

The two main receptors of glutamate, calcium-permeable AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and NMDA (N-methyl-D-aspartate), are also researched heavily with the SOD1-G93A model and collectively represent 21% of the SOD1-G93A Excitability literature. AMPA receptors lacking GluR2 expression have high calcium permeability and are therefore more susceptible to motor neuron death from excitotoxicity (51). Loss, decrease or immunoreactivity of glutamate transporters (56 articles, 19% of Excitability), including EAAT2 (excitatory amino acid transporter-2) (52), all of which have been shown in SOD1-G93A mice, may also give rise to selective motor neuron degeneration.

GABA, another frequent topic in the excitability literature (7% of Excitability), is a neurotransmitter responsible for regulating excitability. Related topics frequently examined include GABA's current density and amplitude (53), concentration (54), release and transmission upon treatments, by itself (55), or with glycine (56), methionine sulfoximine (MSO) (57), ionomycin (58), and HU210 (59).

Besides glutamate and its related measures, the Compound Muscle Action Potential (CMAP) is the next most frequently investigated measure of excitability (35 articles, 12% of Excitability). While modest at first, it has been observed that CMAP amplitudes drastically decrease in the final weeks before death (60).

In perhaps surprising contrast, other forms of SOD1-G93A traditional electrophysiological properties of motor neurons were not in the top 12 or upper 85% of Excitability terms. However, a sector of research is ongoing with, for example, persistent inward currents or PICs (50,61), frequency-current or F-I gain (48,62), and dendritic processing (63), to name just a few examples.

Inflammation. One of the fundamental characteristics in progression of ALS is activation of microglia and astrocytes, a process referred to as neuroinflammation. Inflammation is the second-largest SOD1-G93A pathophysiological category as nearly half of the papers were identified as discussing some aspect of neuroinflammation. An in-depth ontological map of this category is shown in Figure 3. A major goal of inflammation research is to determine which parameters are expediting the disease versus which ones are protecting against it.

The degree of gliosis, scarring caused by reactive astrocytes, is a major indication of ALS progression in SOD1-G93A mice (64), as such reactive inflammatory cells are thought to contribute to death of the motor neurons (MNs). Not surprisingly, nearly half of the most prevalent inflammation key terms is related to some aspect of gliosis. Gliosis is often assessed using GFAP expression (64), which is also the most prevalent key word in the inflammation literature (240 + articles, 39% of Inflammation).

Microglial activation was the second-most assessed inflammatory topic (28% of Inflammation). In particular, common direct measures encompassed cytokines, including iNOS, TNF-alpha, and various interleukins (IL). An increased level of TNF-alpha (11% of Inflammation) has been observed in human ALS patients, but the absence of TNF-alpha in SOD1-G93A mice did not affect the survival (65). Nonetheless, lowering the activity of other cytokines, such as IL-1beta, has been shown to reduce inflammation and extend survival in SOD1-G93A mice (66). Additionally, macrophage activation (9% of Inflammation), which results in the production of cytokines, has been investigated in SOD1-G93A mice and in clinical patients, where an up-regulation is commonly seen (66). Therefore, unsurprisingly,

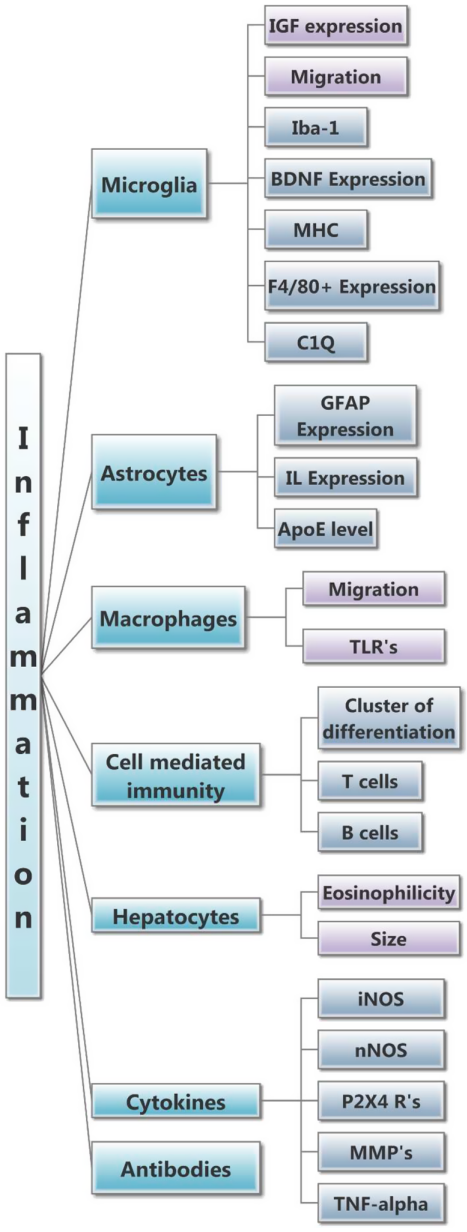

Figure 3. Field map of the inflammation categories based on their pathophysiological properties and significance to demonstrate the variety of terms that were searched. The frequency search reveals that a few measures are used for specific cell types, e.g. GFAP expression was much more heavily used (by 259 articles) compared to ApoE (six articles).

migration of microglia and macrophages is often used to evaluate the effectiveness of anti-inflammatory treatments (67).

Vascular endothelial growth factor (VEGF), which has been investigated in 44 articles (7% of Inflammation), plays a role in neuronal protection from ischemic and hypoxic damages (68), and, used as a treatment, has shown promising results in both SOD1-G93A mice and in humans (69). Although VEGF has been classified under Inflammation due to its protective effects on neuroinflammation, studies suggest it may also act to reduce excitotoxicity and downstream apoptotic pathways (68).

Oxidative Stress. Oxidative stress, representing 19% of the SOD1-G93A literature, reflects an imbalance between the systemic manifestation of reactive oxygen species and the normal physiological ability to readily detoxify the reactive intermediates or to repair the resulting damage (30). Disturbances in the normal redox state of cells, such as those in SOD1-G93A transgenic mice, can cause toxic effects through the production of peroxides and free radicals. Resulting damage can affect many components of the neural and glial cells, including proteins, lipids, and DNA (9). Furthermore, some reactive oxidative species act as cellular messengers in redox signaling. Thus, oxidative stress can interfere with normal mechanisms of cellular signaling, and so many oxidative stress articles also examine their effects on other ontological categories, including excitability, inflammation and systemic outcomes.

Peroxides, and specifically hydrogen peroxide (H_2O_2), represent 29% of the SOD1-G93A oxidative stress literature. Peroxides are produced during the electron leakage from mitochondria (9). Thus, most such articles focus on reducing the effects of peroxides produced by damaged mitochondria (70,71).

Other free radicals and oxidants for which the effects of various treatments have been investigated include nitric oxide (NO-) (72) and peroxynitrite (ONOO-) (73). Transcription factor Nrf2 (6% of Oxidative stress) is known to interact with the antioxidant-response element enhancer sequence to increase protein expression involved in antioxidant defense (74). In the SOD1-G93A model, a significant decrease has been cited in the expressions of antioxidant response genes regulated by Nrf2 (75) and the effects of the Nrf2/ARE system activation (76). Other highly cited antioxidants include glutathione and peroxidase, which were found in over 75 + SOD1-G93A articles (33% of Oxidative stress).

Proteomics. The cellular stress caused by aggregates of mutant, misfolded proteins is a hallmark of neurodegenerative diseases including ALS (36). The misfolded and aggregated proteins are considered to play a lead role in the pathophysiology of the SOD1-G93A transgenic ALS mouse. Hence, the majority of papers in proteomics, 168 or 40% of all proteomics-labeled articles, were concerned with aggregation. Mutant SOD1 seems to impair the proteasomal pathway and autophagy of the cell's degradation machinery (36). Thus, instead of undergoing degradation, these misfolded proteins begin to aggregate in the cell. Like the amyloid tangles in Alzheimer's disease, a major question of the proteomics field has been whether these misfolded aggregates are a cause of the ALS pathology, in and of themselves, or if they are simply a pathological side-effect (77).

Misfolded protein degradation is dependent on proteasomes (54 papers, 13% of Proteomics), which have been shown to be impaired in SOD1-G93A mice (78). The two most common proteasomes studied are LMP2 and LMP7, mentioned by eight and six papers, respectively (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1047455). Ubiquitin (89 articles, 21% of Proteomics) labels proteins for degradation, and has also been shown to be inappropriately included in aggregates (10).

Many patients with ALS show inclusions containing ubiquitinated and phosphorylated TAR-DNA binding protein 43 (36). Most of the articles, 23 in total, examined the amount of TDP-43 as a measure of proteomic progression in SOD1-G93A ALS mice. Additional stress factors resulting from protein aggregation, such as overexpression of various heat shock proteins (HSP) and ER stress (54 articles, 13% of Proteomics), are increased in SOD1-G93A mice and are thought to coincide with disease progression (79). The effect of disulfide-linking at cysteine sites has been shown to slow the rate of mutant SOD1 degradation (80) and subsequently increase aggregation (81).

Systemic. The systemic category includes measures that examine the disease on a higher physiological scale including overall tissue death, functional outcomes, and other possible contributors of non-neuromuscular origin. Additionally, disease onset and endpoint measures are contained within the systemic category. Systemic evaluations are important to SOD1-G93A research because they demonstrate the point at which the pathology is impacting overall function and/or health. Thus, it is not surprising that the systemic category is the most frequently assessed SOD1-G93A ontological category (see Figure 1). Many articles utilize systemic measures to assess their possible relationship to primary experimental measures from other ontological categories.

Rotarod performance (a motor function test where the mouse is placed on a rod rotating at either a constant or accelerating speed and time is measured until the animal falls), grip strength, and grip endurance, are all in vivo physical motor function tests that are utilized to assess neuromuscular disease progression in transgenic SOD1-G93A ALS mice (3). Collectively, these functional measures account for approximately 28% of the systemic category.

Neuronal density and overall motor neuron count, which are known to continuously decrease with SOD1-G93A ALS mouse disease progression, are the most prevalent measures of the systemic category, encompassing 41% of the systemic literature. Unlike the functional measures, they can be measured in either in vivo or in vitro experimental settings.

Systemic measures are also used to assess the disease onset and endpoint in in vivo experiments with SOD1-G93A transgenic ALS mice. Measures of onset appear in greater than 98% of articles assigned to the systemic category. Onset is typically defined by the start of a wobbly gait, hindlimb clasping, or a percent drop in rotarod performance (82). The endpoint is typically defined as either full limb paralysis, failure to stand on a rotating rotarod, or death (3,79).

The least represented sector of the systemic category is articles examining potential disease contributors of non-neuromuscular origin. Only a handful of articles were found. Examples include the role of possible liver disease (83) and T-lymphocytes (84). More research is needed in this non-neuromuscular sector given recent clinical findings citing the high relevance and relationships to overall health and/or other antecedent disease (85).

Categorical relationships and future directions

ALS has long been considered a multi-factorial disease (86). This characterization is supported by the many biological and pathological connections between the different presented ontological categories of the SOD1-G93A ALS pathophysiology. Hence, it is not surprising that each primary data article is typically represented by 2–3 different ontological categories. For instance, excitability (electrophysiology, channels, neurotransmitters, etc.) cannot be adequately studied without considering its strong ties to energetics (ATP, mitochondrial calcium homeostasis, etc.) and axonal transport (transport of mitochondria and neurotransmitters, etc.). A further complication of such pathophysiological relationships is that they do not necessarily have the same sign or direction for the entire disease duration. For example, axonal transport appears to initially increase, possibly as a compensatory mechanism, prior to later showing deficits (2,5,87). Experimental measurement of relationships, especially temporal cross-category relationships (e.g. relationship of excitotoxicity to energetics) is difficult due to the required longitudinal and combinatorial experimental design (2,88)

Temporal relationships in the high-copy mouse model have already been shown to drastically impact the overall dynamics of the SOD1-G93A ALS pathophysiology (2,48). Recent clinical ALS evidence has suggested possible neuroprotective effects related to pre-onset homeostatic regulation and possibly even hypervigilant regulation (85). Additionally, theoretical analysis has shown that treatments which address underlying system-level mathematical regulatory instabilities, including homeostatic oscillations, are more promising than traditional single-mechanism strategies (2,23). Collectively, current evidence indicates that future experimental and informatics analysis of within-category and

cross-category SOD1-G93A pathophysiological temporal relationships is needed to help solve the many remaining mysteries.

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Supplementary material available online

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CHAPTER 3

SEEKING HOMEOSTASIS: TEMPORAL TRENDS IN RESPIRATION, OXIDATION, AND CALCIUM IN SOD1 G93A AMYOTROPHIC LATERAL SCLEROSIS MICE

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Seeking homeostasis: temporal trends in respiration, oxidation, and calcium in SOD1 G93A Amyotrophic Lateral Sclerosis mice

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Impairments in mitochondria, oxidative regulation, and calcium homeostasis have been well documented in numerous Amyotrophic Lateral Sclerosis (ALS) experimental models, especially in the superoxide dismutase 1 glycine 93 to alanine (SOD1 G93A) transgenic mouse. However, the timing of these deficiencies has been debatable. In a systematic review of 45 articles, we examine experimental measurements of cellular respiration, mitochondrial mechanisms, oxidative markers, and calcium regulation. We evaluate the quantitative magnitude and statistical temporal trend of these aggregated assessments in high transgene copy SOD1 G93A mice compared to wild type mice. Analysis of overall trends reveals cellular respiration, intracellular adenosine triphosphate, and corresponding mitochondrial elements (Cox, cytochrome c, complex I, enzyme activity) are depressed for the entire lifespan of the SOD1 G93A mouse. Oxidant markers (H_2O_2, 8OH2′dG, MDA) are initially similar to wild type but are double that of wild type by the time of symptom onset despite early post-natal elevation of protective heat shock proteins. All aspects of calcium regulation show early disturbances, although a notable and likely compensatory convergence to near wild type levels appears to occur between 40 and 80 days (pre-onset), followed by a post-onset elevation in intracellular calcium. The identified temporal trends and compensatory fluctuations provide evidence that the "cause" of ALS may lay within failed homeostatic regulation, itself, rather than any one particular perturbing event or cellular mechanism. We discuss the vulnerabilities of motoneurons to regulatory instability and possible hypotheses regarding failed regulation and its potential treatment in ALS.

Keywords: ALS, motor neuron disease, mitochondria, oxidative stress, Ca^{2+}, energy metabolism

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Introduction

Amyotrophic Lateral Sclerosis (ALS) is a late-onset neurodegenerative disease consisting of progressive muscle atrophy, muscle paralysis, dysarthria, dysphagia, and dyspnea. While there has been much research conducted on the disease, the precise causes and effective treatments have remained elusive. Transgenic mice, and namely the superoxide dismutase 1 glycine 93 to alanine mutation (SOD1 G93A), have served as the predominant means by which to investigate the underlying cellular pathophysiology (Pfohl et al., 2015). A multitude of categorical disturbances have been identified, which are described in detail in a recent informatics-based systematic review

of the SOD1 G93A field (Kim et al., 2015): apoptosis, including changes in pro- and anti-apoptotic signals; energetics, including mitochondrial dysfunction, adenosine triphosphate (ATP) depletion, and calcium homeostasis; excitability, including hypoexcitability, hyperexcitability, and excitotoxicity; genetic transcription, including damage to mRNA and DNA; inflammation, due to reactive microglia and astrocytes; oxidative stress, from the production of free radicals; proteomics, including the build-up of mutant protein aggregates and reduced autophagy or proteasome function; and systemic function, which also includes potential non-neuromuscular contributors.

This Frontiers Research Topic and present study focuses on a unique and highly inter-related triad of the ALS pathophysiology: the role of mitochondria, oxidative stress, and altered calcium homeostasis. Most of the previous experimental work has focused on identifying the presence of impairments in this triad using a wide variety of specific methods and measures, such as recording of the mitochondrial potential, evaluation of intracellular ATP concentration, electrophysiological assessment of calcium entry, and measurement of intracellular free radicals. While the presence of deficiencies in mitochondria, oxidative regulation, and calcium homeostasis has been well established, their timing, as a function of ALS disease initiation and progression, is less understood. The goal of this work is to evaluate their overall temporal trends, pre-natal through death, in the SOD1 G93A transgenic ALS mouse model. In this systematic review of 45 articles, we aggregate *in vitro*, embryonic, and *in vivo* experimental measurements of cellular respiration, mitochondrial mechanisms, oxidative and anti-oxidative markers, and intracellular calcium in both SOD1 G93A transgenic ALS mice and in wild type control mice. We evaluate the magnitude and statistical trend of these assessments in SOD1 G93A mice compared to wild type mice and examine changes over the course of the SOD1 G93A mouse life span.

Materials and Methods

The general method includes: (1) identifying and recapturing published experimental data for SOD1 G93A transgenic mouse and wild type control mouse assessments of mitochondrial function, oxidative stress, and calcium homeostasis; (2) Normalizing recaptured data to temporally compare the assessed measures; (3) Performing a Gaussian average to temporally interpolate the values and develop a visual trend line for each measure and category of measures.

Inclusion and Exclusion Criteria

Potential articles were identified under the PubMed search criteria of (G93A OR transgenic mouse) AND (ALS OR "Amyotrophic Lateral Sclerosis"). Initial exclusion criteria consisted of: non-English language articles; articles for which full-text pdf downloads were unavailable (see Data Recapture); and articles labeled as literature reviews.

Keyword searches of recaptured figure captions and within figure text were performed to find relevant articles from the initial literature pool (see Data Recapture) using the following terms: calcium and its permutations, including Ca^{2+}, Ca, etc.; mitochondria and its permutations (mito*); oxidative stress and its permutations (oxid*), reactive oxygen species (ROS), free radicals, hydrogen peroxide (H_2O_2), nitric oxide (NO), malondialdehyde (MDA), 8-hydroxy-2′-deoxyguanosine (8OH2′dG), heat shock proteins (HSPs). Study-specific inclusion criteria required the use of both non-treated SOD1 G93A and wild type transgenic mice for a given quantitative experimental measurement. "Non-treated" consisted of controlled experimental assessments of the measured parameter(s) without the application of chemicals or processes meant to intentionally attempt to modify the assessed measures or related physiology.

Data Recapture

Articles were either downloaded using PubMed Central or from e-journal subscriptions available from the libraries of Georgia Institute of Technology and Emory University. Data was recaptured from the following article locations (Kim et al., 2015), referred to as entities: article title; abstract; figure captions; within figure text (x–y axis labels, bar graph categorical labels, legends, etc.); and data series and response values (e.g., quantifiable figure/table data). Data was scraped from the full-text pdf article. Every data point was reviewed by an independent quality control team to insure complete accuracy.

Categorical Definitions

Given that each study utilizes its own specific measures, aggregation is a requirement in order to obtain a meaningful visualization of categorical trends and meta-analysis results. Experimental measures were aggregated into four main categories: calcium regulation, cellular respiration, mitochondrial mechanisms, and oxidative regulation. Sub-categories of measures are further defined within each category as discussed below.

Calcium Regulation

The calcium regulation category includes experimental assessments of intracellular calcium dynamics, including calcium entry, calcium sinks, free calcium concentration, and calcium sensitivity. Calcium entry encompassed electrophysiological measurements of extracellular calcium entering through the membrane of motoneuron cells (e.g., Ca^{2+} persistent inward current, Ca^{2+} amplitude, Cell Voltage, Cellular Calcium, etc.). Calcium sensitivity included measures of mitochondrial membrane sensitivity to a discretely measured calcium challenge. Calcium sink values encompassed measures of calcium buffering or calcium capacity. Calcium concentration encompassed general cytosolic free calcium levels.

Cellular Respiration

The cellular respiration category includes measures of general respiration rate as well as intracellular concentrations of ATP and adenosine diphosphate (ADP). Measures of the general respiration rate include production of heme, oxygen consumption, or respiration control ratio.

Respiration Mechanisms

Respiration mechanisms included experimental assessment of complex I activity, COX activity, Cytochrome C levels, and general mitochondrial enzyme activity (including Complex III, IV, V). These parameters are involved the electron transport chain (ETC), which leads to the production of ATP.

Oxidative Regulation

Experimentally assessed contributors to oxidative stress include hydrogen peroxide (H_2O_2) production and the oxidant markers MDA and 8-hydroxy-2′-deoxyguanosine (8OH2′dG). The concentration of HSP was also incorporated in the oxidative regulation category given their neuroprotective effects and potential impact on the slowing of oxidant-induced symptoms. Specific HSPs included HSP 25, 27, and 70.

Tissue Sources

In vivo tissues primarily consisted of SOD1 G93A mouse brain or spinal cord cells; in fact, 35 articles identified at least one of these two regions as a source. Some articles utilized homogenized tissue or multiple tissue sources (Leclerc et al., 2001; Damiano et al., 2006; Johnson et al., 2011; Miana-Mena et al., 2011), which included spinal cord or brain tissue with other systemic tissues, such as blood, liver, soleus, diaphragm, and liver. Tissues used for *in vitro* assessment were more varied. Cell lines mostly included standard SOD1 G93A transfected mice cells. However, other G93A-transfected sources included SH-SY5Y cells (Carri et al., 1997; Goos et al., 2007; Pesaresi et al., 2011), NSC-34 cells (Liu et al., 2002; Ferri et al., 2008; Mali and Zisapel, 2009; Crippa et al., 2010), yeast (Gunther et al., 2004; Kloppel et al., 2010), and bacteria (Singh et al., 1998).

Analysis

The ratio of SOD1 G93A to wild type (e.g., SOD1 G93A/wild type) is used to normalize each assessed measure. Each study was normalized to its own published wild type data. For each included measure or category of measures, the ratio of SOD1 G93A to wild type is plotted versus time. Data from each article is given equal weight. For ease of visualization, all *in vitro* cell line experimental data is plotted as −20 days and embryonic experimental data is plotted as −5 days; *in vivo* data is plotted at its corresponding post-natal day of experimental assessment. A standard Gaussian average was performed to interpolate values in-between the raw experimental data points and to produce trend lines indicative of the general aggregate behavior of each experimentally measured parameter.

Results

In total, 262 data points from 45 unique papers were collected and normalized for inclusion in this meta-analysis. **Table 1** shows

TABLE 1 | Categorization, distribution, and sources of included experimental data.

	Papers	Values	Sources
Calcium regulation			
Entry	2	12	Johnson et al. (2011), Quinlan et al. (2011)
Sensitivity	2	11	Pieri et al. (2003), Damiano et al. (2006)
Sink	6	14	Pieri et al. (2003), Damiano et al. (2006), Igoudjil et al. (2011), Milanese et al. (2011), Pesaresi et al. (2011), Tradewell et al. (2011)
Concentration	9	26	Carri et al. (1997), Kruman et al. (1999), Beers et al. (2001), Goos et al. (2007), Guatteo et al. (2007), Milanese et al. (2011), Nutini et al. (2011), Panov et al. (2011), Tradewell et al. (2011)
Mitochondrial mechanisms			
Complex I	1	3	Loizzo et al. (2010)
Enzyme activity	5	49	Leclerc et al. (2001), Jung et al. (2002), Mattiazzi et al. (2002), Wendt et al. (2002), Gunther et al. (2004)
COX	1	4	Kirkinezos et al. (2005)
Cytochrome C	5	11	Guégan et al. (2001), Jung et al. (2002), Liu et al. (2002), Kirkinezos et al. (2005), Damiano et al. (2006)
Cellular respiration			
Adenosine triphosphate (ATP)	4	21	Browne et al. (2006), Ferri et al. (2008), Mali and Zisapel (2009), Peixoto et al. (2013)
Adenosine diphosphate (ADP)	2	18	Leclerc et al. (2001), Browne et al. (2006)
Respiration rate	7	20	Gunther et al. (2004), Damiano et al. (2006), Cassina et al. (2008), Igoudjil et al. (2011), Panov et al. (2011), Miquel et al. (2012), Zhao et al. (2012)
Oxidative Regulation			
8-hydroxy-2′-deoxyguanosine, 8OH2′dG	1	4	Fang et al. (2010)
Hydrogen peroxide, H_2O_2	5	7	Singh et al. (1998), Liu et al. (1999, 2009), Takamiya et al. (2003), Kloppel et al. (2010)
Malondialdehyde (MDA), $C_3H_4O_2$	5	36	Hall et al. (1998), Liu et al. (1999), Fang et al. (2010), Miana-Mena et al. (2011), Seo et al. (2011)
Heat shock proteins (HSP)	5	26	Vleminckx et al. (2002), Batulan et al. (2003), Maatkamp et al. (2004), Jaarsma et al. (2008), Crippa et al. (2010)

There are four major categories of measures: intracellular calcium, mitochondrial mechanisms, cellular respiration, and oxidative regulation). Sub-categories of measures, as defined in the Methods, are listed under each category along with the number of included unique articles and extracted data points. Each data point represents a paired SOD1 G93A and wild type mouse assessment at a discrete time point.

the experimental data point and article distribution to each of the defined categories and subcategories of measures. The category, mitochondrial mechanisms, which contains measurements of constituents necessary for cellular respiration, includes 67 values from 10 unique articles. Cellular respiration, which includes the assessed respiration rate and intracellular concentrations of ATP and ADP, includes 59 values from 12 unique articles. The oxidative stress category, which includes oxidative markers and anti-oxidative HSPs, has a total of 73 values from 14 unique articles. Intracellular calcium, which contains measures examining intracellular calcium homeostasis, contains 63 data points from 15 unique articles.

Cellular Respiration is Depressed for Entire Lifespan

Among the most interesting trends found in this meta-analysis study is that ATP production, along with general respiration rates, were found to be depressed for the entire lifespan of the SOD1 G93A mouse (**Figure 1**). While it has been well documented that respiration rates are lowered in ALS (Kawamata and Manfredi, 2010; Cozzolino and Carri, 2012; Peixoto et al., 2013) even well before physical pathologies develop (Browne et al., 2006), this meta-analysis supports the assertion that this phenomenon is a trend that, at least in the high-copy SOD1 G93A transgenic mouse model, is present since birth. That is, the SOD1 G93A mice have notable depression of cellular energetics well before symptom onset, and this depression remains throughout the course of disease progression.

Figure 1 illustrates the ratio of transgenic mouse to wild type mouse experimentally measured levels of intracellular ATP, ADP, and respiration rate. All of the *in vitro* cellular data (plotted as −20 days in **Figure 1**) collected for intracellular ATP concentration and respiration rate fall well below their wild type counterparts. Examining the mathematical mean of *in vitro* measures of ATP and respiration rate reveal that SOD1 G93A levels are approximately 70% of those seen in wild type, which is equivalent to a 30% reduction.

Post-natal *in vivo* assessment of intracellular ATP and respiration rate in SOD1 G93A mice also shows substantial depression compared to wild type mice. While ATP and respiration rate is depressed throughout the life span of the SOD1 G93A mouse, there appears to be small fluctuations throughout the disease course. However, more data is necessary to determine whether these small fluctuations are statistically or mechanistically meaningful. ATP is at its lowest at the disease end point, where ATP levels approach only 30% of wild type (**Figure 1**). Interestingly, the temporal trend of ADP is slightly different than ATP and respiration rate. For most of the disease course, ADP is depressed in a similar manner to ATP and respiration. However, ADP levels in SOD1 G93A mice show an interesting rise above wild type control mice that occurs near the disease end point. This near-death rise in ADP could be

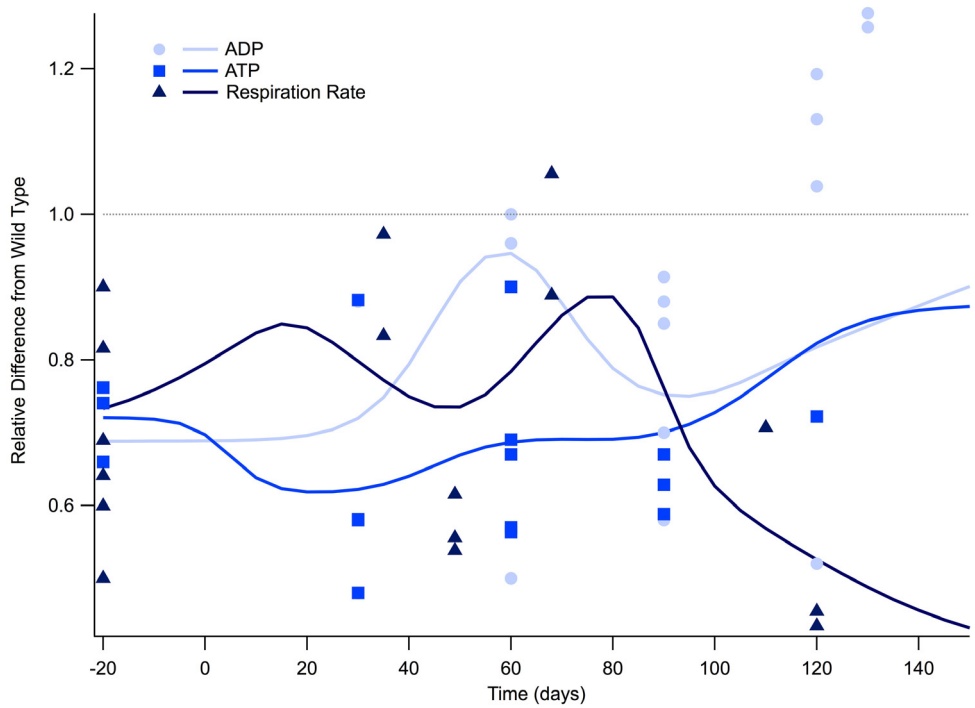

FIGURE 1 | Cellular respiration is depressed for the lifespan of the SOD1 G93A transgenic mouse model. The ratio of SOD1 G93A to wild type (SOD1 G93A/wild type) is plotted over time for experimental measures of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and general respiration rate. Solid lines illustrate predicted trend lines. For visualization purposes, *in vitro* data is plotted at −20 days and *in vivo* time points are plotted at their corresponding post-natal day of assessment, 0–150 days. A gray dotted line is provided to show the expected wild type or homeostatic value. Trend lines are generated based on a Gaussian average of the normalized data points.

attributed to the cells' inability to convert ADP to ATP, which would leave ADP in excess. In fact, this same trend in the lowering of the ATP/ADP ratio is seen in clinical patients (Ghiasi et al., 2012).

Depression of Mitochondrial Mechanisms

Additionally, measures of mitochondrial mechanisms and signals necessary for respiration and ATP production are similarly depressed throughout the course of the disease. **Figure 2A** illustrates the temporal trends of four different mitochondrial mechanism measurements and signals necessary for cellular respiration: complex I, COX, general enzyme activity, and cytochrome C. *In vitro* measures were only obtainable for enzyme activity, which shows a depression similar to that seen in ATP levels. Post-natal *in vivo* assessment of SOD1 G93A mice reveals that all four of the mitochondrial mechanism measurements are generally depressed compared to wild type. The *in vivo* depression is present at birth and throughout the entire disease duration. Although the mitochondrial mechanism experimental measures remain below wild type, the Gaussian average trend lines identify a potential small bump in mitochondrial mechanism activity near disease onset (around 80 days), which could represent a regulatory compensation mechanism; a larger sample size is necessary to determine if this small bump has possible statistical or mechanistic implications in disease progression. Finally, when the mitochondria's ability to produce ATP is impaired, there is a compensatory increase in mitochondrial enzyme complexes, especially Complex II, III, IV (Nalbandian et al., 2015). This upward trend in mitochondrial enzymes is seen **Figure 2A** near post-onset and the disease end point.

Elevation of Oxidants Near Onset

Oxidant levels have been documented as being elevated throughout different stages of ALS (Liu et al., 2002; Mattiazzi et al., 2002; Martin et al., 2007), including pre-onset (around 40 days), onset (80 days), and especially end-stage (120+ days). In **Figure 2B** we plot the temporal trends of three commonly measured oxidants in SOD1 G93A mice (8OH2′dG, MDA, and H_2O_2). The data presented in **Figure 2B** reveals that SOD1 G93A intracellular oxidant levels are initially similar to slightly above wild type at birth. However, by pre-onset, levels are mildly elevated, and at onset and end point, oxidant levels are substantially increased compared to wild type. *In vitro* assessment of oxidants does not reveal as pronounced of elevation as the *in vivo* assessments. *In vivo* assessment of oxidants reveals a 1.5 factor increase in oxidants compared to wild type around symptom onset (80 days). *In vivo* assessment near the SOD1 G93A disease end point (120+ days) reveals oxidant levels that are a factor of 2–8 times greater than seen in wild type control mice.

Heat shock proteins, which have an anti-oxidative effect, are also plotted in **Figure 2B**. HSPs in SOD1 G93A mice were found to initially be substantially greater than wild type levels, but they exhibited a fluctuating decline as the disease progressed. However, there appears to be a recurrent delayed rise in HSP as the disease enters the symptomatic stage (around 80 days).

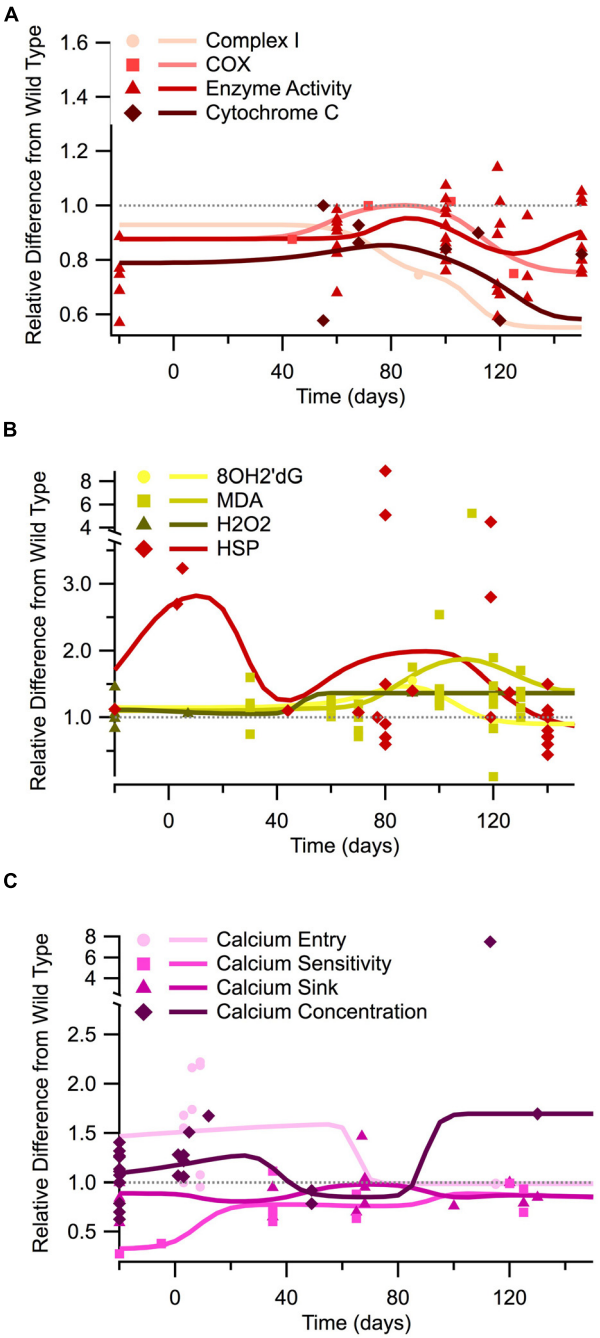

FIGURE 2 | Temporal trends of mitochondrial mechanisms, oxidant regulation, and calcium regulation in the SOD1 G93A transgenic mouse model. The ratio of SOD1 G93A to wild type (SOD1 G93A/wild type) is plotted over time for each experimental measure. For visualization purposes, *in vitro* data is plotted at −20 days, embryonic data is plotted at −5 days, and *in vivo* time points are plotted at their corresponding post-natal day of assessment, 0–150 days. A gray dotted line is provided to show the expected wild type or homeostatic value. Trend lines are generated based on a Gaussian average of the normalized data points. **(A)** Mitochondrial mechanisms (Complex I, COX, enzyme activity, and cytochrome c). **(B)** Oxidant markers (8OH2′dG, MDA, H_2O_2) and protective heat shock proteins (HSPs). **(C)** Calcium regulation (entry, sensitivity, sink, and cytosolic concentration).

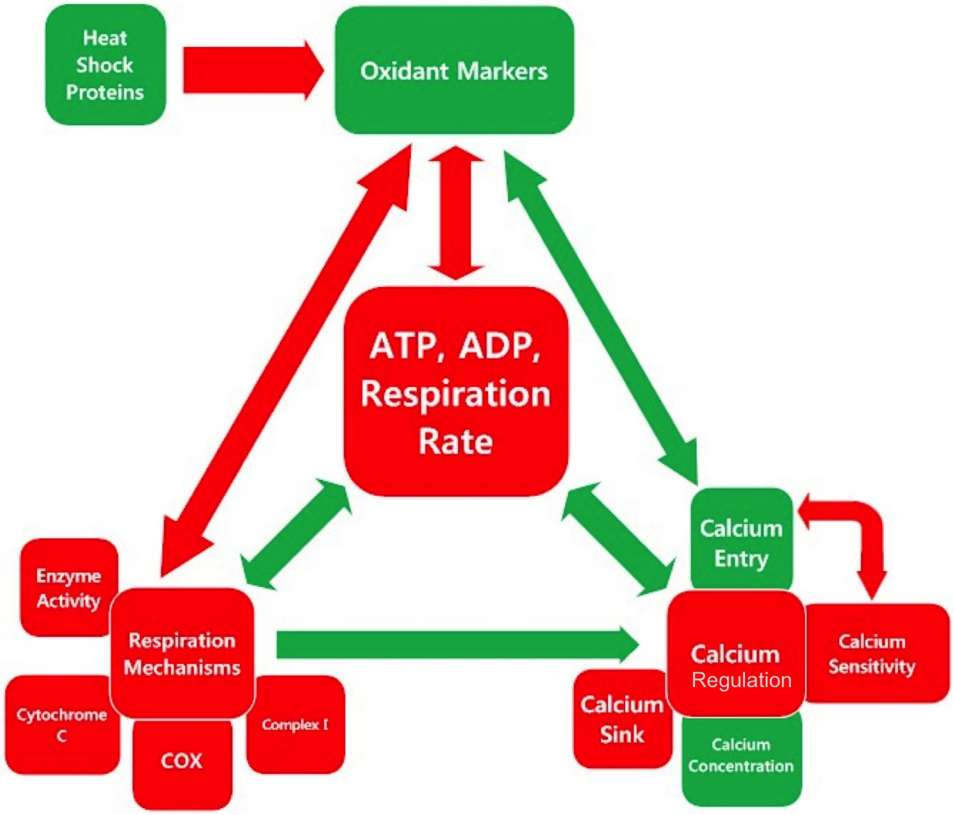

FIGURE 3 | Inter-relationships in the mitochondrial mechanism-oxidative regulation-calcium regulation triad center around cellular respiration. Each square is colored based on whether the assessed category is, on average, higher (green), or lower (red) in SOD1 G93A mice compared to wild type as determined by the overall trend line directionality from 0 to 150 days. The color of the arrow indicates the sign of the relationship, and the size indicates the relative magnitude.

This fluctuation has been previously described. It is hypothesized that HSP levels are insufficient to quell the oxidant rise. Thus, decreased HSP levels actually precede motor neuron loss in ALS (Maatkamp et al., 2004).

Fluctuations of Intracellular Calcium

Calcium homeostasis is critical for both functional neural excitability and normal cellular signaling. There are four main experimental types of calcium regulatory measurements: calcium entry (incoming calcium through ion channels), calcium sensitivity (measurement of the cell's rate of response to calcium), calcium sink (binding and storage of intracellular calcium, including buffers, transporters, and intracellular stores), and the actual calcium concentration (free intracellular concentration). Each of these measures contributes to the balance or homeostasis of intracellular calcium.

In vitro data examining free intracellular calcium concentration is conflicting, with about half of the data points showing elevated calcium and half showing lower intracellular calcium compared to wild type (points plotted at −20 days in **Figure 2C**). These apparent conflicts in *in vitro* intracellular calcium concentration could possibly be explained by the usage of different tissue types for *in vitro* assessment (see Tissue Sources). Measurements of *in vitro* calcium sinks are depressed compared to wild type, ranging from 60 to 80% of that seen in wild type mice, and calcium sensitivity is about 30% of wild type. Post-natal *in vivo* assessment of intracellular calcium and calcium entry at birth reveals levels that are substantially above wild type. However, free calcium and calcium entry appears to dip to near-normal levels during pre-onset. There is limited data for SOD1 G93A mice calcium entry and concentration from onset through end point, but available data revels that calcium appears to rise sharply after onset, resulting in a disease end point intracellular calcium concentration that is a factor 1.5 greater than wild type. *In vivo* assessment of calcium sinks and calcium sensitivity in SOD1 G93A mice show depressed levels compared to wild type from birth through the disease end point. Intuitively, the point at which the calcium sink trend line is highest coincides with the time points when calcium concentration, or free-floating calcium, is lowest.

Discussion

The results of our systematic review and meta-analysis of 45 articles shed new light on the temporal trends of cellular

respiration, oxidative markers, mitochondrial mechanisms, and calcium regulation in the SOD1 G93A transgenic ALS mouse model. By aggregating data, we show that cellular respiration and corresponding mitochondrial mechanism are impaired for the entire lifespan of the SOD1 G93A mouse. Oxidant markers are initially similar to wild type but are more than double that of wild type by the time of symptom onset despite early post-natal elevation of protective HSPs. All aspects of calcium regulation show early disturbances, although a notable and likely compensatory convergence to near wild type levels occurs between 40 and 80 days, which is followed by a divergence after symptom onset.

This systematic review clearly shows that SOD1 G93A mice exhibit a long-term metabolic deficit, however, these symptoms are also present in other ALS mouse models. Dupuis et al. (2004) performed metabolic experiments on both G93A and G86R mice to demonstrate the similarities in mitochondrial function. G37R mice also show significant reduction in ATP production (Coussee et al., 2011). Finally, substantial metabolic disturbances have also been documented in non-SOD transgenic mice, including mice with mutations in TDP-43, FUS, VCP, among others (Carri et al., 2015).

Interactions within the Respiration-Oxidation-Calcium Triad

There are multiple feedback loops between the triad of cellular respiration, calcium regulation, and oxidative regulation. The inter-relationships between the categories and sub-categories of measurements examined in this meta-analysis are illustrated in **Figure 3**. Red boxes indicate parameters, which are lower in SOD1 G93A mice compared to wild type, and green box indicates parameters, which are higher in SOD1 G93A mice compared to wild type. Similarly, the color of the arrows indicates either a positive relationship (green) or a negative relationship (red), and their size indicates the relative strength of the relationship. The biology of these interactions is summarized below.

Mitochondria have a highly interactive dynamic with the endoplasmic reticulum (ER), the main intracellular calcium storehouse. Mitochondria take up calcium via the calcium-sensitive mitochondrial uniporter. However, sustained free cytosolic calcium inactivates the uniporter, preventing further calcium uptake (Moreau et al., 2006). Accumulated calcium in the mitochondria can then be released back into the cytosol via the sodium–calcium and hydrogen–calcium exchangers (Fuchs et al., 2013). Once intramitochondrial calcium rises above a certain level, the mitochondrial transition pore opens, initiating apoptotic or necrotic signaling cascades (Leung and Halestrap, 2008). Calcium originating from the activation of AMPA receptors and/or pathologically increased membrane permeability is thought to result in this shift of calcium from the ER to the mitochondria. Ryanodine receptors on the surface of the ER further amplify calcium-mediated calcium release from the ER, which in turn, could further exacerbate AMPA activation (Berridge, 2002). A second receptor that exacerbates calcium release from the ER is the calcium-activated IP3R.

Collectively, intracellular release of calcium from the ER, mitochondria, and other intracellular stores could explain the increase in intracellular cytosolic calcium concentration seen near onset (∼100 days), which is mirrored by a paradoxical decrease in extracellular calcium entry (see **Figure 2C**). Another contributor for this apparent paradox could be a decrease in expression of calcium binders like calbindin D28K and parvalbumin (Celio, 1990), which have been proposed to result in increased cytosolic calcium in ALS mice (Appel et al., 2001). Chin et al. (2014) similarly shows a decrease in parvalbumin in SOD1 G93A mice as well as a reduction in sarcoplasmic/ER Calcium ATPase proteins, including SERCA1. Notably, calcium binders, which fall under the calcium sink category in this meta-analysis, show a slight dip that also corresponds to the timing of the intracellular calcium increase (**Figure 2C**).

Calcium release has a bi-directional relationship with ROS production since ROS homeostasis is maintained via Ca^{2+} signaling and Ca^{2+} dependent pathways. Calcium stimulates NO synthesis and leads to ROS production at Complex III (Feissner et al., 2009). Moreover, because the ryanodine receptor forms a tetramer with the sarcoplasmic and ERs, the reversible oxidation of endogenous superoxide groups can result in the release of additional calcium from the sarcoplasmic reticulum (Fill and Copello, 2002). Finally, oxidative agents like peroxide directly induce calcium release from the ER via IP3R (Wesson and Elliott, 1995). In summary, free radicals induce calcium leakage into the cytosol via the ryanodine receptor, Ca^{2+}-leak channels, and inositol 1,4,5-trisphosphate receptors, and conversely, intracellular calcium concentration activates NOX and NOS, which then produces additional excess ROS and RNS, respectively (Chin et al., 2014). Ultimately, elevated internal calcium creates a cyclical feed forward mechanism that continually increases calcium and oxidative stress to the point of apoptosis (Chin et al., 2014).

Lower respiration rates, and consequently, lower intracellular ATP concentrations, directly contribute to the lowering of the mitochondrial potential, which can ultimately initiate apoptotic cascades. The impact of lower ATP concentration on oxidative and calcium imbalances is bi-directional, with increases in oxidants and calcium-mediated calcium release further impairing mitochondrial function, especially Complex 1, a key constituent for ATP production (Cassina et al., 2008; Cozzolino and Carri, 2012; Lautenschlager et al., 2013). Through a less direct path, an increase in oxidative stress can also lead to a swelling of mitochondria, which also further inhibits ATP production (Martin et al., 2007). Finally, lower concentrations of ATP impede calcium-ATPase in removing free calcium from the cytosol or shuttling calcium back to the ER for storage (Kaplan et al., 2003; Fuchs et al., 2013).

Deciphering the Timing: Cause, Effect, and Instability

Because of their large size and innate emergent properties, motoneurons are susceptible to homeostatic instabilities. It has been previously shown that motoneurons, even in a physiological state, have insufficient mitochondrial capacity to buffer large calcium fluxes. Calcium buffering insufficiency is thought to be due to a reduced mitochondrial density per

volume compared to non-motoneurons (Grosskreutz et al., 2007). Therefore, mitochondrial dysfunction and impaired calcium homeostasis is hypothesized to account for the selective vulnerability of motoneurons (Jaiswal and Keller, 2009; Jaiswal et al., 2009). Another contributor for the selective vulnerability of motoneruons is the requirement for axonal transport of mitochondria over very long distances, up to 1 m (Mitchell and Lee, 2009, 2012a; Lee and Mitchell, 2015). Finally, the dynamics of somatic input processing of motoneurons could explain the earlier death of fast twitch fibers in ALS (Mitchell and Lee, 2011).

Because there are so many interacting variables, it is difficult to determine which parameter(s) initiate versus simply affect the pathophysiological cyclical cascades of depressed cellular respiration, imbalances in calcium homeostasis, and intracellular elevation of oxidants in ALS. The presented data reveals that elevated oxidants appear later in the SOD1 G93A life span, closer to disease onset. However, both calcium and cellular respiration/mitochondrial mechanisms show early deficits. Much like the age-old question, "What came first, the chicken or the egg?" This meta-analysis begs the question, "What comes first—improper calcium homeostasis or depressed cellular respiration?"

Scientifically justified arguments could be made for either position. Increased calcium permeability or ATP depletion from sub-par cellular respiration, or a combination of both, could initiate a dynamic instability in the motoneuron that results in the ALS phenotype. The trend lines presented in this meta-analysis reveal the presence of potential compensatory mechanisms, which attempt but ultimately fail, to re-stabilize to homeostasis. For example, between 0 and 20 days, there is a rise in HSPs and a gradual increase in calcium sensitivity. The "slight bump" in mitochondrial mechanisms/cellular respiration at pre-onset also coincides with the lowest intracellular calcium levels. Attempts to re-stabilize to homeostasis could potentially correspond to the small fluctuations apparent in **Figures 1** and **2,** although more data is needed to definitively determine their statistical significance.

Mathematical instabilities within pathophysiological feedback loops have already been identified in a dynamic meta-analysis of the SOD1 G93A mouse model (Mitchell and Lee, 2012b). If unstable pathology dynamics are the actual underlying culprit, it may not actually matter exactly which mechanism first initiated the cascade (see Future Directions). Both preclinical and clinical failures to obtain meaningful success using single-mechanism treatments illustrate the potential validity of this point. Among the many examples are: dichloroacetate (Miquel et al., 2012), which attempts at restoring the mitochondrial respiratory capacity in the astrocytes, *N*-acetyl-glucagon-like peptide-1 (Sun et al., 2013), which endogenously regulates metabolism by promoting insulin synthesis and secretion, and creatine (Groeneveld et al., 2003; Snow et al., 2003; Beal, 2011), which is known to enhance ATP synthesis. However, no single treatment to targeting cellular energetics has been effective enough to translate to an effective treatment for humans (Tadic et al., 2014).

In this meta-analysis, we reveal that, although there are some small fluctuations, cellular respiration is depressed for the entire SOD1 G93A ALS mouse lifespan. Interestingly, ALS patients, prior to the onset of their ALS, have been found to be healthier (e.g., less antecedent disease) than age, gender, and geography-matched control subjects (Mitchell et al., 2015). However, such patients could still have asymptomatic pre-ALS variations in their underlying motoneuron regulation, which make them more susceptible to instabilities. Hypervigilant regulation as been put forth as one possibility to explain how the above-average pre-ALS health of patients could be correlated to a later, destabilizing motoneuron perturbation, which initiates ALS (Mitchell et al., 2015). "Hypervigilant regulation" results when underlying regulatory processes aggressively overreact to correct imbalances from homeostasis, making them 'hypervigilant' to perturbation (in control theory, referred to as a too-high feedback gain). While hypervigilant regulation would initially be overall protective, it could also result in greater later susceptibility to destabilization, especially in highly susceptible motoneurons (Mitchell et al., 2015). The temporal calcium, oxidant, and HSP fluctuations identified in this study, in combination with the oscillatory behavior of other previously identified parameters (Mitchell and Lee, 2012b) such as axonal transport (Mitchell and Lee, 2012a) and excitability (Delestree et al., 2014), are suggestive of the possible role of regulatory and homeostatic impairments as being the "cause" of ALS.

Future Directions

Perhaps instead of focusing on mechanistic initiation, treatments should focus on treating the underlying instability, itself (Mitchell and Lee, 2008, 2012b). Whether in engineering process or in biology, treating instability typically requires impacting multiple targets or feedback loops, which may or may not have directly initiated the destabilizing perturbation or event. Combination treatments can leverage synergistic interactions to increase treatment effect size. Multiple experimentalists have attempted combinatory treatments on the SOD1 G93A mouse model (Waibel et al., 2004; Feng et al., 2008; Del Signore et al., 2009; Del Barco et al., 2011). For example, Waibel et al. (2004) experimented with rasagiline, an anti-apoptotic with neuroprotective properties, combined with riluzole, a sodium channel blocker, to reduce excitotoxicity. The combinatory treatment did exhibit a statistically significant improvement compared to control and compared to Riluzole alone.

In addition to exploiting synergistic interactions to increase effect size, combination treatments could potentially be used to re-stabilize the system to homeostasis. Theoretical SOD1 G93A ALS models of combination treatments have shown this exciting possibility (Mitchell and Lee, 2012b). In fact, of the several thousand computationally assessed combination treatment permutations, a few percent of 2 and 3-way treatment strategies were able to mathematically stabilize the ALS pathophysiology (Mitchell and Lee, 2012b). Interestingly, energetics was one of the pathophysiological categories that most frequently appeared in synergistic or stabilizing treatment combinations. Given the early and lasting depression of ATP and respiration rates identified in the present study, it is not surprising that energetics was previously predicted to have the greatest single-category effect size (Mitchell and Lee, 2012b).

Based on the results of the present study, it would appear that therapeutics leveraging the strong interactions within the calcium-respiration-oxidation triad could be promising. As shown in **Figures 1** and **2,** prior to onset, it appears the SOD1 G93A physiology temporarily compensates toward decreasing intracellular calcium, increasing anti-oxidative HSPs, and slightly increasing respiration rate; thus, treatment to amplify these existing compensatory mechanisms would seem intuitive. However, like spinal cord injury (Mitchell and Lee, 2008), such treatments would likely have to be initiated very early in the disease process to have a meaningful effect. In fact, in the case of instability, the timing of treatment may be the most important parameter, especially given human patients will not be treated until after ALS symptoms appear. Obtaining a finer point on the timing and statistical significance of fluctuations in intracellular calcium, ATP concentration, and free radicals, is critical to devising combination treatments that have clinically significant results. An additional essential research path is better assessment of homeostatic regulation. Modulation of regulatory pathways may prove more fruitful for re-stabilization than direct physical or chemical manipulation of cellular elements.

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CHAPTER 4

UNDERGRADUATE BIOCURATION: DEVELOPING TOMORROW'S RESEARCHERS WHILE MINING TODAY'S DATA

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ARTICLE
Undergraduate Biocuration: Developing Tomorrow's Researchers While Mining Today's Data

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Biocuration is a time-intensive process that involves extraction, transcription, and organization of biological or clinical data from disjointed data sets into a user-friendly database. Curated data is subsequently used primarily for text mining or informatics analysis (bioinformatics, neuroinformatics, health informatics, etc.) and secondarily as a researcher resource. Biocuration is traditionally considered a Ph.D. level task, but a massive shortage of curators to consolidate the ever-mounting biomedical "big data" opens the possibility of utilizing biocuration as a means to mine today's data while teaching students skill sets they can utilize in any career. By developing a biocuration assembly line of simplified and compartmentalized tasks, we have enabled biocuration to be effectively performed by a hierarchy of undergraduate students. We summarize the necessary physical resources, process for establishing a data path, biocuration workflow, and undergraduate hierarchy of curation, technical, information technology (IT), quality control and managerial positions. We detail the undergraduate application and training processes and give detailed job descriptions for each position on the assembly line. We present case studies of neuropathology curation performed entirely by undergraduates, namely the construction of experimental databases of Amyotrophic Lateral Sclerosis (ALS) transgenic mouse models and clinical data from ALS patient records. Our results reveal undergraduate biocuration is scalable for a group of 8-50+ with relatively minimal required resources. Moreover, with average accuracy rates greater than 98.8%, undergraduate biocurators are equivalently accurate to their professional counterparts. Initial training to be completely proficient at the entry-level takes about five weeks with a minimal student time commitment of four hours/week.

Key words: biocuration, text mining, database, biomedical informatics, bioinformatics, neuroinformatics, health informatics, data science, lab management, big data, undergraduate research

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Defined by the International Society for Biocuration, biocuration involves the translation and integration of information relevant to biology or medicine into a database or resource that enables integration of the scientific literature as well as large data sets. The primary goal of biocuration is to accurately and comprehensively present integrated data as a user-friendly resource for working scientists and as a basis for computational analysis. There has been rapid expansion of published literature, experimental data, electronic clinical records, and continuous health monitoring data. The curation of biomedical data has become a necessity to explore new problem domains using informatics analysis, a field more recently referred to as "big data." In fact, because of the magnitude and breadth of available data and the development of innovative informatics analysis techniques, biocuration is quickly becoming one of the most invaluable research aids.

Curation of any kind of biomedical data or literature is typically considered a Ph.D. level task due to the integrated level of knowledge required to classify complex information (Burge et al., 2012). While automated tools exist to expedite the process, biocuration currently remains a largely manual and time-consuming task. The level of education and the required manual labor and time are critical reasons why there is a massive shortage of biocurators (Burge et al., 2012). On the other hand, the number of undergraduates wishing to obtain research experience and crucial career-enhancing data analytical skills is nearly unlimited. To this end, we have developed a biocuration process that enables undergraduates to successfully curate biomedical data at accuracy and productivity rates that equal professional curators.

Our approach includes partitioning the complex biocuration process into digestible steps that collectively encompass a serial assembly line, which can be operated by a "small army" of undergraduate biocurators, from 8-50+ per semester. By dispersing the workload, each undergraduate curator works a reasonable but effective four hours/week, thereby preventing burnout. Another important part of undergraduate biocuration success is the creation of an undergraduate lab environment that closely resembles a business structure with a hierarchy of curator, technical, managerial, and administrative positions. Curation is the foundational layer of the hierarchy. However, the business structure creates additional opportunities for undergraduate skill set development for both academic research and traditional industry careers while simultaneously alleviating the managerial time commitment of the primary investigator (PI)/professor.

In this article, we outline the process utilized to initiate and maintain an undergraduate biocuration assembly line. This method is ideally suited to informatics/theoretical labs or primary investigators/programs who wish to establish undergraduate research positions suitable for students headed either into academic research or industry. Smaller-scale versions could also be envisioned in wet/experimental/clinical labs that have their own large

data sources that they wish to curate. Compared to many research projects, biocuration requires very little start-up financial or physical resources while still offering amazing career-transforming opportunity for undergraduates. Moreover, the developed curated database products and enabled informatics analysis is invaluable to biomedical data science.

Because our lab's primary area of expertise is computational analysis of neurophysiology and neuropathology, all of our biocuration has focused on neuropathology of disease (Amyotrophic Lateral Sclerosis, Alzheimer's Disease, Spinal Cord Injury). However, this method is adaptable to any experimental (in vitro, in vivo, physiology or pathology) or clinical data set.

METHODS

Biocuration involves first establishing a data path as well as developing a human workflow. Below we summarize the required resources and the data path process and go into detail on how an assembly line of curators and hierarchical undergraduate research positions can be utilized as a "undergraduate curation corporation."

Required resources

Other than a data source and eager undergraduate students, the physical resources needed include basic computers with standard office productivity software, database software, a database server, and a secure local area network from which the database server runs such that users can simultaneously enter data into a remote database. A quiet purpose-specific environment is preferable. Security of the database and the environment must be assessed depending on the type of data being curated (e.g., clinical data curation requires many more security protections compared to experimental data). For our ALS informatics project, curation was done on project-specific computers in a room with a closed door with multiple layers of environmental (controlled access) and computational security (multiple logins, firewalls, etc.). Resources required for automated and/or manual database back up must also be in place.

Establishing a data path

Establishing the data path includes instituting the process from accessing the data to database development to curating the data to the ability to analyze the data. Note that, while initially serial, a data path is an iterative loop. That is, there are always more refinements as the curation project progresses. Bicourators of different sub-fields each have their own personal preferences for establishing a data path. Here we summarize the activities for establishing a data path that we believe work best for undergraduates.

Data source. The first step to developing undergraduate biocuration is to establish one or more large data sources to begin curating. Data sources could be clinical medical records, unpublished experimental data, or published experimental data. When establishing a data source, simply keep in mind that the point of biocuration is to pool and organize data sets into a form that is both useable to other researchers and can be used to perform bioinformatics analysis. Also, determine that all protocols for permission and approval to data access (e.g., Internal Review Board for clinical data, etc.) are in place.

Data pool. The next step is to establish what parts of the data source will be curated. Being as inclusive as possible is usually the best option. More metrics allow more analytical options and more complex research questions to be pursued. Quantitative metrics are the most preferred, but parametric data is also valuable. Any data that has pervasive commonality (e.g., a survey that all patients have in common) or can be standardized or normalized (e.g., a biomedical experiment that includes a wild type or control) should definitely be included.

Alpha curation. Before the development of a database, a group of alpha testers employ individualized manual methods to collect entries from the data pool. Collecting data in a database program or even a simple spreadsheet is acceptable. Having multiple testers helps to include multiple points of view. These test curators, with the PI, establish the data fields and the initial anticipated workflow.

Establish database. Based on the alpha curation, a relational database is created that allows entry, review, and easy export of curated data for statistical analysis. We use the Filemaker (Filemaker, Inc.) database software, because, in our experience, it is more friendly and intuitive for novice database users, both in terms of layouts (graphical user interfaces) and in scripting language. However, Access (Microsoft, Inc.) or any other preferred database platform could be utilized.

Beta curation. Before the database is released for official curation by a large group, beta testers not involved in the construction of the database use the database to identify bugs, suggest edits in design, or suggest addition of automation/user tools to reduce error. The database is refined based on beta curator input.

Workflow. The order in which the data is curated and how it is formatted must be explicitly specified. The development of written protocols is key to insure data homogeneity and integrity.

Primary curation. Primary curation is the bulk of curation performed as part of the main project or database construction.

Data quality control. Establishing manual and automated data quality control procedures is critical for insuring data integrity. These procedures will vary slightly depending on the curated data type. More detail is given in the Quality Control section under Positions.

Classification. After a substantive amount of primary curation, classifications (such as functional ontologies) can be imposed to assist in searching, aggregating, and analyzing data. We recommend designing for both a universal ontology, to be used by any database user, and user-specified ontologies, to be used and customized by specific tech teams (see Positions) as part of their analysis.

Prioritization. For very large biocuration projects, developing a prioritization scheme can be helpful to insuring that the most important or time-sensitive data or data relevant to the pilot projects' goals is curated first.

Meta-data analysis. Meta-data simply means "data on the data." Meta-data of curated data in the database is

used to determine sample sizes, which specify the feasibility of a pilot project's goals.

Feasibility study. A feasibility study is the process of evaluating potential pilot project alternatives based on meta-data, scientific or clinical significance or user need.

Pilot project. A pilot project is a research project that utilizes the curated database for exploratory or hypothesis driven research or to develop a product. Depending on available sample sizes and timelines, it may or may not be a full-size study. Some pilot projects can develop into full-size studies once proof-of-concept has been obtained.

Applicants

Applicants range from high school juniors through senior undergraduate students. As shown in Figure 1, our applicants come from a variety of majors, including all different types of engineering, biological science, chemisty, computer science, business/finance majors, and many others. Irrespective of major, most applicants seek us out due to their perceived interest in neuroscience or medicine. Because we are in the biomedical engineering (BME) department, the majority of applicants are BME majors. Many of the BME majors and especially the non-BME majors have a pre-health track, which means they have had requisite exposure to basic biology knowledge and quantitative skills necessary for curation. Applicants complete our lab's official application and submit a one-page resume. The application is a combination of questions, including questions to assess personality, attention to detail, interests/extracurricular activities, classwork, special skill sets, future career plans, and perhaps most importantly, a paragraph written by the applicant stating why they desire a position. An in-person interview by the PI is utilized to assess intangible qualities.

Unlike many labs seeking undergraduates, we don't simply target "the crème de la crème" with the highest GPA, etc. In fact, we have found that GPA is not even a good predictor of success in a biocuration and informatics environment. Desire, commitment, consistency, attention to detail, and fundamental biological and quantitative knowledge (e.g., how to read a graph or an experimental protocol, etc.) appear to be the essential basic applicant qualities at the entry-level. Every person starts at the same position so the students sort themselves out in the hierarchy.

Initial Training

As shown in Figure 1, there are three phases of initial training: introductory lectures, project training and curation training. Introductory lectures are classroom-style teaching sessions where associates are given a primer on biocuration, an overview of basic neuroscience, and fundamental scientific and/or clinical knowledge on the topic/condition/pathology being assessed. Interactive or hands-on project training teaches the applicants everything they specifically need to know about the project, especially the types of measures being curated and their definitions, and any ethical or research protocols for handling the data. For example, most of our clinical ALS informatics project associates had at least a one-day rotation at an ALS Clinic

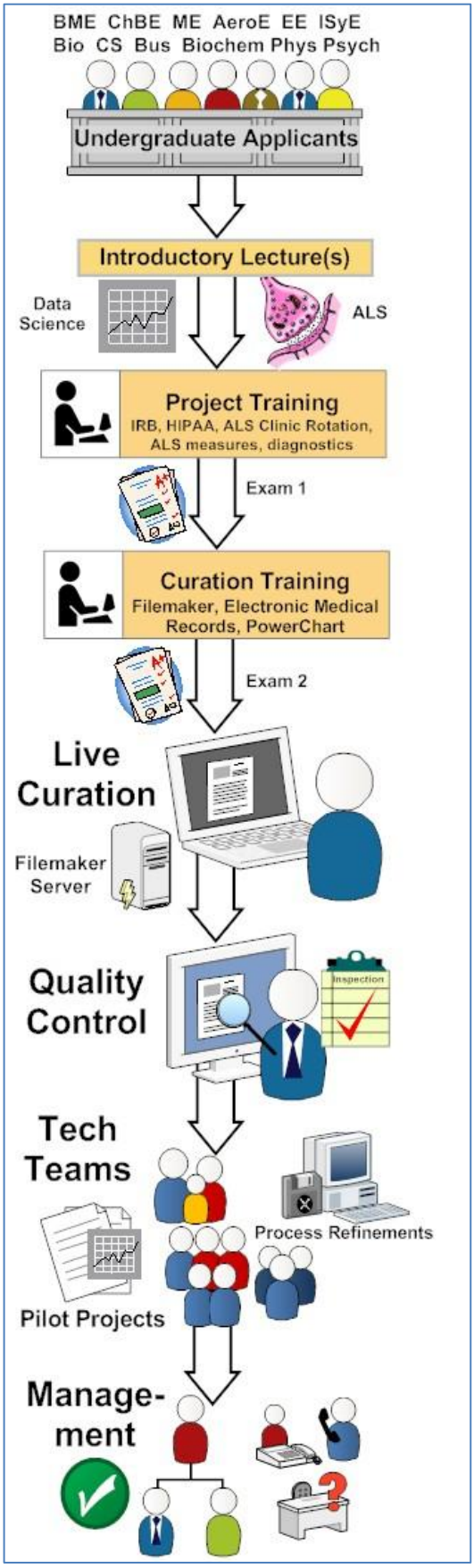

Figure 1. Application, training, and job positions. ALS clinical informatics project used as a detailed example.

to help them better understand the information that was being curated in the chart. Interactive or hands-on curation training teaches the applicant everything they need to know in terms of data transcription—how to access the data, how to use the database software, how the database is organized, and ultimately how to find/search, view, edit and add new data

Exams testing concept recall immediately follow each training session. The curation training session also has a practical exam where students curate specified data into their own individual practice database for a period of one week. A score of 95% is required to pass the practical exam. Individualized tutoring and up to two retakes are allowed before dismissal. Upon passing the practical, students undergo a competency assessment, which is a four-week period of live curation in the actual project database. If the associate shows good promise in meeting the quality control productivity and accuracy standards and has consistently worked their specified schedule, they are finally offered a contracted position.

Positions

Curators: Curation is our foundational and entry task/position. Curators collect/transcribe data and enter it into the project-specific database. Whether an associate is a senior in high school or a senior in college, he/she will begin as a curator. The amount of expertise required for curation will vary by project. For very complex data paths and/or to lower the experience barrier for applicants, curation can be divided into different "levels" that form a serial assembly line. Curators are promoted to each subsequent level as they gain the necessary experience and training and prove their desire and skill. For example, our SOD1 G93A ALS transgenic mouse informatics projects has multiple levels of curators: 1) document capture—Level 1 associates access and save the data files (in this case, published articles) in an organized, appropriate format for subsequent transcription; 2) figure capture—associates transcribe figure captions by panel. 3) data series capture—associates transcribe non-interpretative experimental parameters names, experimental methods, mouse genotypes, etc.; 4) response value capture—associates transcribe quantitative data, which may or may not require subjective interpretation by reading data from a figure, etc.; 5) informatics capture—capturing additional project-specific data, applying organization or ontological schemes, or even doing meta-data analysis. In essence, these curators serve as consultants to the technical teams.

Despite detailed initial training, there are always questions that occasionally surface in regards to specific entries. We developed a "curator que" that uses a freely available online interactive classroom website (Piazza), which we have adapted for posting questions/answers, reminders, and tips. Quality control managers respond to posted questions within 24 hours.

Most curators work a reasonable but effective 4-6 hours/week. This amount of workload is perfect for associates who are assessing their interest. Moreover, in an assessment of curator workload, we have determined that 4-6 hours/week results in optimal productivity and accuracy. Curation is a very detail-oriented task, and this must be considered when determining the workload (hours worked and required productivity). In fact, we prefer that associates work in multiple two-hour intervals versus a continuous four or six hours. Online scheduling is utilized to manage computer access, which optimizes computer resource requirements and insures curators always have a computer when they come in to the lab to work their scheduled time periods. Our typical curator tenure is about three semesters, with a percentage of students being promoted to tech teams at the end of semester two. However, many students work as a curator much longer than the average simply because they enjoy the task or the time required fits their schedule while still satisfying their desire to participate in research.

Quality Control (QC): Just like any manufacturing process or company, the purpose of quality control is to ensure that the product (i.e., the database) and performed service (i.e., biocuration) adheres to a defined set of quality criteria. Our QC personnel quantify curator productivity and accuracy, and provide weekly feedback and required corrections. Quality control personnel are typically highly skilled former curators who wish to obtain real-world management experience. As such, many have aspirations to do industry research and development project management. Most QC personnel work eight hours/week, although the minimum is four hours/week.

A written quality control protocol specifies how automated script checks and visual inspection of entries should proceed for each data source and curation type. This insures consistency among QC personnel. The lead QC manager also monitors the efficacy and consistency of QC personnel via random visual inspection and automated statistical analysis. The training of the quality control team is similar to the biocuration training: it consists of a hands-on workshop, a concept exam testing QC protocol knowledge, and a competency exam assessing their performance of entries. The QC personnel to biocurator ratio ranges from 1:5 to 1:10, depending on the degree of complexity of the curation and the amount of available automation as part of the QC process.

The quality control process, itself, consists of the QC personnel using a combination of automation and visual inspection to check curated entry accuracy and curator productivity. For every single entry in the curated database, there is an associated scribe ID, creation time stamp, and modified time stamp so that we can track when the entry was made, modified, and who completed it. Each week, quality control personnel are randomly assigned a list of associates' work to check. Automation works well for finding most missing entries or fields, typos/mis-spellings, assessing qualitative entries, and checking for formatting. Automation also works well for calculating productivity (entries completed over a specific time period). However, to determine if quantitative data is entered correctly or if all possible data has been gathered from the data source, QC personnel must visually inspect and compare the curator's entry to the data source. The QC personnel complete a feedback form for each curator detailing their mistakes and

positively commenting about good performances (e.g., accurately entering a very complex figure, etc.) as appropriate. The feedback cites the database field keys for each entry that requires modification. The curators have one week to fix identified mistakes before re-checking by QC personnel.

For each curation level, there is an associated requirement for productivity and accuracy. Prior to corrections, we set the required curator weekly accuracy standard to be 97.5%. This rate was based on accuracy standards referenced by other academic and industrial curation projects using Ph.D. biocurators (see Results). Nonetheless, our actual accuracy rate prior to correction is 98.8%. Productivity requirements vary by curation level and project. We utilize beta tests during data process development to determine productivity standards. The general rule of thumb is that the productivity standard is 2 standard deviations below the beta test average. This method ensures that the productivity standard provides ample motivation to be efficient but is also sufficiently low so as to not rush or penalize a curator for doing a thorough job or taking a little time to learn about data along the way.

A point-based system is used to track productivity and accuracy for both penalization and reward. Penalty points for not meeting quality control standards are used for performance grading and determining readiness of promotion (see Performance Review).

Technical Teams: About 60% of our curators go on to join a technical or "tech" team. Tech teams consist of 2-5 students who perform small pilot data science projects using curated data. Projects may be exploratory or hypothesis-driven. All teams start by assessing meta-data and creating any necessary classification or ontologies. Teams are typically paired with one or two curator consultants to expedite getting the data in the form the team needs for their specific project. In our lab, exploratory studies utilize complex systems-based techniques to identify relationships within the data and testable hypotheses. Traditional hypothesis-driven studies typically consist of large-scale meta-analyses or the use of standard statistics to answer a specific question. Tech teams, on average, require three semesters to complete a published article although we have had several extremely motivated teams submit an article or conference proceeding after two semesters. A few teams also develop data science tools to improve and automate our curation process.

Tech team project penultimate deliverables or outcomes can vary based on both the PI's desires (ongoing projects and deadlines) and the team members' desires (long-term career goals like authoring an article in preparation for graduate school, development of specific skills for industry, planned tenure in the lab, school schedule, etc.). Typically, we have had the best success if the penultimate deliverable was a useable product/tool by the lab or public, a published conference proceeding, or a peer-reviewed journal article. Such deliverables encourage and externally motivate not only the tech teams, but also the curators. Some continuity within teams (i.e., one or more team members seeing the tech team project to completion) is preferred but is not necessarily a requirement with proper documentation and, if possible, a hand-off or transition period that includes start-up training and/or an apprenticeship by at least one of the new team's members.

The primary investigator provides one or more broad topics, questions, or product goals from which the team can pursue to choose. However, it is up to the tech team to shape the chosen path into successful project. A senior-level experienced undergraduate technical team manager handles day-to-day management of tech teams, which have pre-set weekly intermediate deliverables set by the PI. Initially, tech teams are given tasks that include project-specific curation combined with intense literature searches or the equivalent thereof to become proficient on the background of their project's topic. Common technical skills (how to use reference software, export data, make a figure, scientific writing, etc.) are taught in sessions led by tech team managers. After ascertaining a knowledge background, the focus then shifts to activities beyond the primary curation data process (see Establishing the data process). The first semester concludes with a feasibility study assessing pilot project alternatives, one of which the tech team ultimately pursues in the next semester.

After semester one, a tech team is analogous to a graduate student fulfilling his/her research proposal. Tech team managers are still available to teach specific common statistical/analytical skills (meta-analysis, ANOVA, cox proportional hazards, Kaplan-Meir, etc.) and to oversee day-to-day productivity and intermediate deliverables. However, the PI/professor oversees the tech team project direction as the research becomes more project-specific, especially during the construction and formatting of the penultimate deliverable.

Information Technology (IT) Team. The IT team is really just another specialized form of a tech team whose focus is on enhancing IT aspects of biocuration rather than performing analytical informatics on a particular research question. Thus, some of our associates with high-end computer skills or IT-related career plans choose to join our IT team instead of a traditional research tech team. The IT team refines the database and develops automation to enhance curation workflow, quality control, and pilot project informatics analysis. They may also serve as consultants to tech teams by assisting in writing scripts or other programming tasks. Finally, the IT team is responsible for all IT maintenance, including weekly back-ups, login accounts, servers, hardware and software.

Management. As noted in the above sections, there are undergraduate managers for curation quality control, IT, and tech teams. Technical team managers, who are senior-level students, oversee the daily activities of the technical teams; lead skills training sessions (using reference software, making figures, scientific writing tips) and provide consulting for statistical and informatics analysis. QC managers direct the curator que (online system for asking curation questions), maintain our training and QC protocols, and oversee the curator and quality control personnel master schedules. Our IT managers coordinate and oversee all of the IT team's activities.

Our managers are mostly advanced students that have

been with us for the majority of their undergraduate tenure. Minimally, they must have completed all levels of curation. In fact, QC or IT managers are only required to have previously completed curation. However, undergraduate tech team managers must have completed curation and at least one and preferably two semesters as a tech team member. Managers may also be technicians or graduate students. The commonality among all of the managers is that they oversee the day-to-day activity of their core group and the submission of weekly and monthly productivity reports. They are also the primary point-of-contact to the PI/professor. Managers are mostly trained through an apprenticeship system, although our lab does have specific written protocols in place regarding the responsibilities of each managerial role.

Performance Review

As noted in the quality control section, a point-based infraction system is used to grade curator accuracy and productivity. A similar system is used to grade technical team and manager productivity via the use of weekly or bi-weekly intermediate deliverables. All students are given contracts at the beginning of each semester that outline the expectations for their position(s) and the equivalent points which translate to a specific performance grade. Students are given weekly feedback on their performance, so there are no surprises. Students must maintain a "B" to have their contract renewed the following semester. Consistently poor performance during a semester results in demotion to a lesser position (for example, a Level 2 curator may be demoted to Level 1). If productivity standards for the demoted position are not met, the student's contract is ultimately rescinded. A total of three communicated warnings precede dismissal. Dismissal is done in an in-person meeting with the PI.

On a more positive note, Biocurator-of-the-Month awards, which include a small prize and a publicly displayed certificate on the lab door, are given to the most productive (e.g., entries/hour) biocurator(s) who also did not incur penalty points for excessive errors. Analogously, technical-associate-of-the-month awards recognize outstanding performance by tech team members or managers.

Compensation

During the initial training and competency assessment period (approximately six weeks), students are uncompensated. For their first three semesters with a position, most students take optional research course credit at the rate of 3-4 working hours per credit hour, depending on position type. For subsequent semesters, managers and exceptional technical team members may receive hourly pay if PI funds are available. Many of the exceptional student researchers apply for and receive independent research funding through the university's undergraduate research program (Georgia Tech President's Undergraduate Research Award) or other outside similar programs. In general, curators are not given authorship on articles, although in special cases, Level 5 curators who serve as consultants may be considered. Authorship is generally reserved for tech team members, and is discussed as part of the initial tech team establishment.

Primary Investigator Commitment

As one might expect, leading an "undergraduate biocuration corporation" can be a substantive time commitment to the primary investigator. Nonetheless, the commitment is manageable with PI scheduling forethought. The greatest amount of time is spent with the initial set-up (writing of the curation and management protocols, determining data sources and possible technical project topics, and interviewing/training the first batch of students). This phase temporarily requires a full-time commitment by the PI; for example, a summer semester might be an ideal time to start a program. After initial set-up, the steady-state operation of the overall program is most directly proportional to the number of advanced technical teams, which require the most input on behalf of the PI. As a point of reference, approximately two advanced technical teams, properly and personally mentored by the PI, take the equivalent PI time investment as a full-time graduate student. PI oversight of data source biocuration fluctuates as a function of project phase and especially the undergraduate quality control management experience. On average, eight fully trained and mentored curators require approximately the same PI time commitment as one full-time graduate student.

RESULTS AND DISCUSSION

We have developed our process using three different experimental disease models: spinal cord injury (Mitchell and Lee, 2008), ALS (e.g., Mitchell and Lee, 2012; Irvin et al., 2015; Kim et al., 2015; Mitchell et al., 2015; Coan and Mitchell, 2015; Pfohl et al., 2015), and Alzheimer's disease (Foley et al., 2015). As part of our biocuration protocol and database development, we have utilized over 350 different undergraduate curators and 10 high school curators. One of the major pros of an undergraduate biocuration program is that it is scalable. We started with a team of three alpha curators. We went through four major scale-ups, about one per year. We currently maintain a total team of 50+ per semester, which includes about 30-40 primary curators and 15-20 tech team members and managers. Based on our experience, to run and easily maintain a true curation assembly line with both curators and full-time quality control, at least eight students are necessary. The addition of a couple of tech/IT teams and managers brings the minimum total for a "curation corporation" to be around 15 students. The maximum number of students is simply a function of student interest, physical and data resources, and of course PI/professor time.

Case Study: Clinical health informatics

By curating ALS Clinic medical records, we developed a novel clinical ALS database, which consists of over 300 different quantitative and qualitative measures, including pre-ALS health, ALS progression metrics, clinical treatments, diagnostic tests, and autopsy reports. The pilot project included 300 patients, and the completed database

includes an astounding 1,587 patients, the largest and most comprehensive ALS data set available to date. Such databases make way for epidemiological studies of demographics, disease progression and treatment. For example, our resultant comprehensive assessment of antecedent disease, which found that ALS patients have substantially less other disease compared to matched non-ALS controls, has resulted in novel hypotheses regarding possible neuroprotective mechanisms (Mitchell et al., 2015). Other examples of published related tech team projects include the identification of novel autopsy pathological marker relationships (Coan and Mitchell, 2015). Currently, an additional five tech teams have ongoing projects utilizing the clinical ALS database.

Case Study: Experimental model informatics

Our largest database is our ALS transgenic mouse database, which curates quantitative data from 3,500+ articles and 35,000+ figure panels into ~50,000 different metrics and treatments assessed over 160,000 time points. Since the inception of tech teams a couple of years ago, 12 ALS tech teams and two AD tech teams have published six peer-reviewed journal articles and eight conference proceedings to date. Numerous other articles are in review or in preparation. Examples of hypothesis-driven tech team projects include: meta-analysis examining the relationship between amyloid beta and mouse cognition (Foley et al., 2015) and sex-dependent progression patterns in SOD1 G93A mice (Pfohl et al., 2015). Examples of exploratory data science tech team projects include: informatics-based analysis of the SOD1 G93A field topics to develop a functional ontology (Kim et al., 2015) and assessing novel homeostatic instabilities in ALS metabolism (Irvin et al., 2015). Finally, the curated products (i.e., the databases), themselves, are an invaluable researcher resource. Our first release of the searchable SOD1 G93A ALS mouse figure database is available on our website: http://www.pathology-dynamics.org. Ongoing work continues.

Undergraduate curators are productive

Eager undergraduates are very productive. Because curation consists of much more than copy and paste transcription, the required capacity and opportunity to learn about the topic being curated maintains interest. The quantified productivity of biocuration obviously depends on the complexity of the data being curated. As a reference, an undergraduate curator reading through paragraphs of unorganized dictated text from medical records of standard clinic visits can transcribe, on average, about 10 layouts per hour of about the size and complexity shown in Figure 2 (ALS Clinical Informatics layout).

For an experimental data capture project, Table 1 illustrates the curation rates for different levels of data capture from published primary experimental data articles (SOD1 G93A ALS mouse). The actual rate of productivity varies by the amount and type of data in each article. Generally, capturing all qualitative and quantitative data from an article takes less than two hours.

Undergraduate curators are accurate

Our biocuration accuracy requirement is 97.5% and is based on published tolerance of error in similar projects by professional biocurators (Keseler et al., 2014; Wu et al., 2014a; Wu et al., 2014b). However, for our SOD1 G93A transgenic ALS mouse experimental database, which curates published in vivo and in vitro data, our actual accuracy (based on 4+ years of biocuration quality control calculations) is an astounding 98.8% with a per-semester standard deviation of 0.2%.

Of the average 1.2% of entries recognized by quality control as erroneous, only 10.5% are classified as “critical errors,” which meaningfully compromise the integrity of the data. Thus, our *effective accuracy* is 99.9%. Discounting the fact that the ALS clinical informatics database does not require reading quantitative values from graphs, the accuracy of biocuration in that database is very comparable.

Our average error rate of 1.2% is in line with other similar databases that employ professional curators. For example, the Candida Genome Database (CGD) and the EcoCyc Escherichia Coli Database employ manual biocurators that are Ph.D. biologists. The CGD has an overall error rate average of 1.82% (Keseler et al., 2014). The EcoCyc Escherichia coli database has an overall error rate average of 1.40% (Keseler et al., 2013). Thus, utilizing a curation assembly line, undergraduates are very capable of doing professional quality biocuration.

Figure 3 shows the breakdown of error types for the SOD1 G93A ALS mouse database for full data capture (aggregated curation for levels, 1-4). The largest percentage of errors is ontological labeling errors (the placement of tags to make finding data easier). Labeling of ontological terms requires the most knowledge of the SOD1 G93A ALS pathophysiology. Ontological labeling is also the only subjective or interpretative entry in the database. Thus, it is not surprising that ontological labeling has the highest error rate. Fortunately, ontological labeling errors do not in any way affect the integrity of the curated data, itself. Partial data capture errors are the second-most common error. Finally, about 11% of all errors are estimation errors, which are quantitative interpretative errors from reading a graph. Almost all critical errors, which affect data integrity, are the direct result of over- or under-estimation of quantitative values.

Automation and Future Directions

Recently, we have been utilizing automated ontological scripts to assign ontological tags established on the presence of certain key words in relevant fields. Based on one semester of data, this method appears to be substantively reducing this error type. To reduce partial data capture errors, we have also recently added automated field checks, which appear to be very effective in reminding curators to fill in required fields. We are also in the process of testing freeware to estimate values in-between tick marks on graphs embedded in pdf files. Finally, automation is also being employed to calculate productivity using computerized time stamps. Our IT team

continues to pursue projects to enhance automation in the entire data path process, including curation, quality control, and analysis.

Biocuration enhances career opportunities

One of the major benefits of biocuration in comparison to more traditional undergraduate research opportunities is that it opens up research to a greater number of students with more varied skill and/or career desires. Curation teaches basic data organization and analytical skills necessary for any career. It also serves as an equalizer, giving all students uniform opportunity to climb the

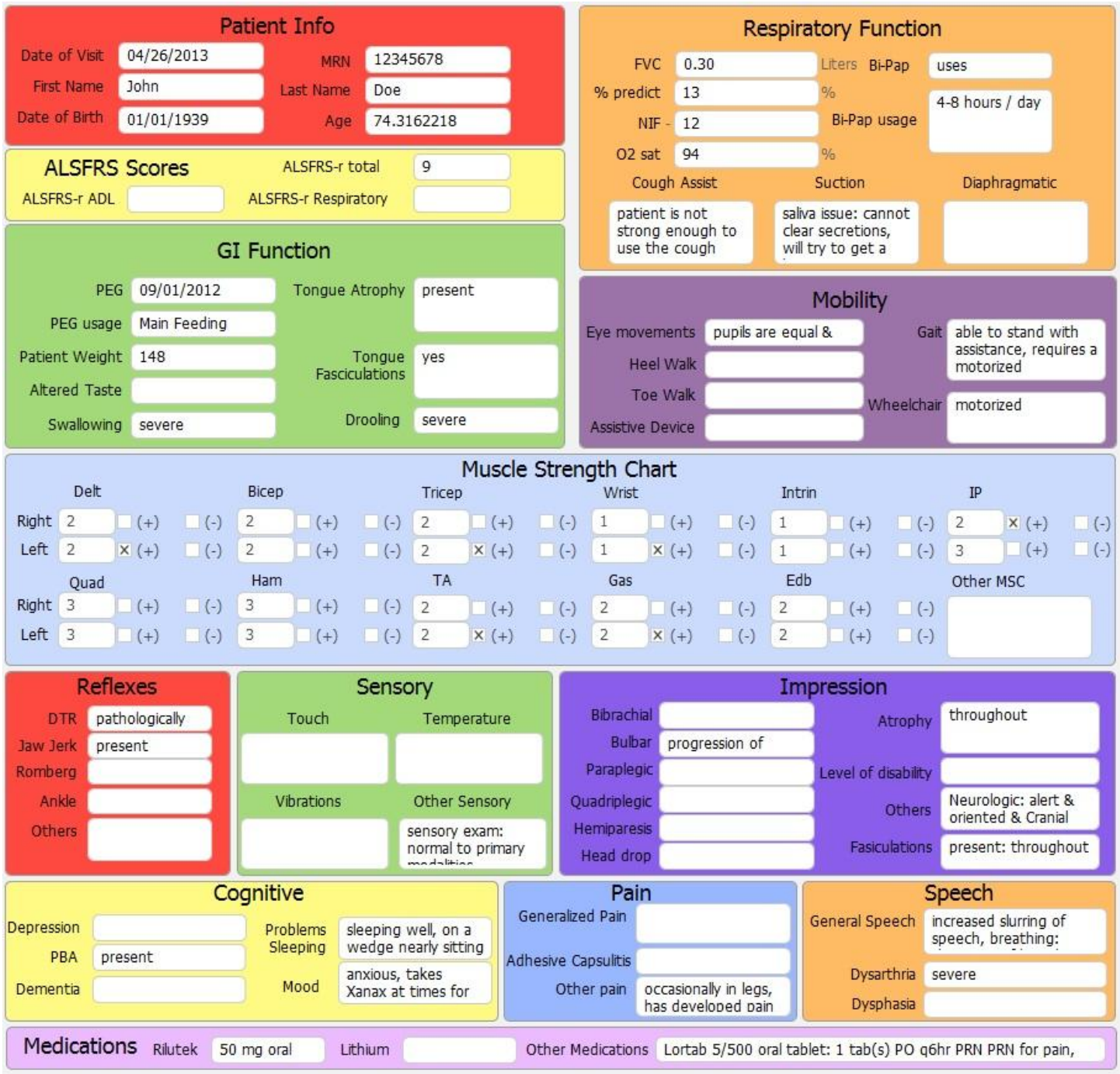

Figure 2. Example curation data entry layout from ALS clinical informatics project. This is one of the four data entry layouts used as part of our ALS clinical informatics project. The layout above shows some of the parametric and non-parametric data that is recorded during a standard ALS clinic patient visit. Additional separate layouts (not shown) exist for cognitive testing, patient history (medical and family history, onset symptom timeline, diagnostic and genetic testing), and autopsy and pathological reports. If no data was present in the medical record or survey for a particular field, the field is simply left blank. Note that patient name and MRN fields are only shown for reference as to how data is obtained from data source; curated data is ultimately de-identified to protect patient privacy.

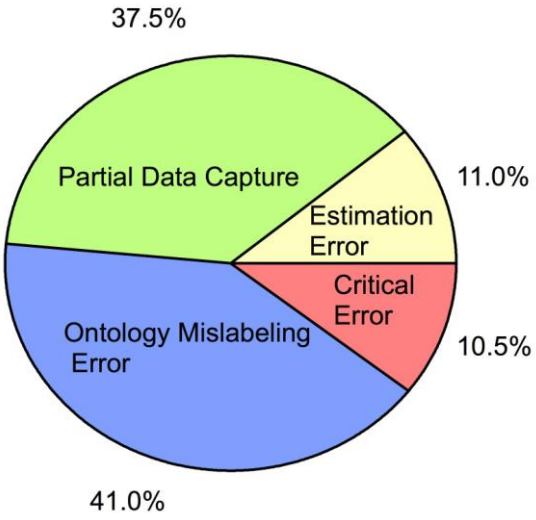

Figure 3. Pie chart illustrating curator error types for Full Data Capture (Curation Levels 1-4, see curation section in Positions) for our SOD1 G93A transgenic mouse ALS experimental database as identified by quality control personnel. On average, trained curators commit errors on less than 1.2% of their entries with a standard deviation of ± 1%. The pie chart represents the breakdown of error type of this 1.2% of total errors. Partial Data Capture *(green)*: curator fails to collect all data from a figure or table. For example, trendline data was recaptured only for G93A and not for G93A + treatment. Ontology mislabeling error *(blue)*: curator assigns response value entry to wrong ontological classification. Estimation error *(yellow)*: captured data point value (typically from a graph) is visually estimated as greater than ± 5% from the actual value. Note that value estimation off by more than 10% is defined as a critical error. Critical error *(red)*: incorrectly entered data that compromises data integrity.

Capture Type	Average Time (per article)	Curation Description
Document	2 to 3 min.	Download full-text PDF document from GT library or PubMed Central
Figure	10 to 25 min.	Extract figure/table captions, panel description, experimental type
Data Series	30 to 60 min.	Extract data series types: mouse strain & attributes, treatments, significance
Response Values	60 to 120 min.	Extract experimental parameters & numeric data from quantifiable figures
Full Capture (total)	*100 to 200 min.*	*All of the above.*

Table 1. Biocuration capture type descriptions and entry times for published experimental model (e.g., SOD1 G93A ALS mouse).

hierarchy. Technical teams favor students that intend to pursue graduate school, academia or research-focused jobs. Management and IT tend to favor students headed into industry or project management. Formal end-of-semester forms are used to track the students' undergraduate research and post-graduate career plans throughout their undergraduate tenure in the lab. Additionally, informal exit surveys are utilized to track post-graduation positions and acceptance. Based on this data for about 350 students, we have determined that a tenure of three or more semesters was analogous to a 0.4-point GPA boost in the very competitive biomedical engineering industry, including student co-op/internships and post-graduation job offers. A management position is analogous to 0.6-point GPA boost for biomedical industry, and about one-third of our multi-semester managers were offered industry project management positions. To date, 80% of students who authored a research publication or proceeding and applied to graduate school or professional school have been admitted.

Conclusions

Undergraduate biocuration can be successfully utilized to develop large, powerful databases and analyze corresponding informatics data. Undergraduate biocurators using the assembly line curation method described have accuracy and productivity comparable to professional Ph.D. biocurators. Moreover, biocuration provides invaluable research experience to a broader population of students who may not otherwise obtain a research position or hands-on experience. Because of the breadth of positions involved in biocuration, it utilizes many different skill sets which are applicable to both research and industry jobs.

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